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POLYGENETIC DIVERSITY OF FREE-LIVING NITROGEN FIXING BACTERIA ISOLATED FROM DIVERSED ECOSYSTEMS OF THAILAND

Miss Orawan Piyaboon

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2000

ISBN 974-7359-77-4

POLYGENETIC DIVERSITY OF FREE-LIVING NITROGEN FIXING BACTERIA ISOLATED FROM DIVERSED ECOSYSTEMS OF THAILAND

Suranaree University of Technology Council has approved this submitted in partial fulfillment of the requirements for the Master's Degree

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อรวรรณ ปิยะบุญ : ความหลากหลายทางพันธุกรรมของแบคทีเรียตรึงไนโตรเจนแบบอิสระในระบบนิเวศวิทยาที่ต่างกันของประเทศไทย
(POLYGENETIC DIVERSITY OF FREE-LIVING NITROGEN FIXING BACTERIA ISOLATED FROM DIVERSED ECOSYSTEMS OF THAILAND)
อาจารย์ที่ปรึกษา : ศ.ดร. นันทกร บุญเกิด, 137 หน้า ISBN **974-7359-77-4**

ความหลากหลายทางพันธุกรรมของแบคทีเรียตรึงไนโตรเจนอิสระที่แยกจากดินในระบบนิเวศวิทยาที่ต่างกันจาก 3 ภาคของประเทศไทย ได้แก่ ภาคเหนือ ภาคกลาง และภาคตะวันออกเฉียงเหนือ โดยในแต่ละภาคจะเก็บตัวอย่างดิน 11 พื้นที่ที่มีระบบนิเวศต่าง ๆ กัน การศึกษาปริมาณประชากรของแบคทีเรียตรึงไนโตรเจนอิสระโดยวิธี serial dilution จากตัวอย่างดินแล้วนำมาทดสอบเพาะเลี้ยงบนอาหารแข็งที่ไม่มีแหล่งอาหารไนโตรเจนแล้วมีการคัดเลือกเชื้อแบคทีเรียตรึงไนโตรเจนอิสระ โดยมีการศึกษาดังนี้ 1) ลักษณะรูปร่างและสรีรวิทยา 2) ประสิทธิภาพการตรึงไนโตรเจน 3) ลักษณะทางชีวเคมี และ 4) การเพิ่มปริมาณ DNA โดยวิธี PCR ชนิด *nif* D และ ERIC ผลการทดลองพบว่าเชื้อแบคทีเรียทั้งหมดย้อมติดสีแบบแกรมลบ ส่วนมากเป็นแกรมลบรูปร่างแบบแท่ง 63.51% และแกรมลบรูปร่างแบบแท่งสั้น 36.49% สำหรับประสิทธิภาพการตรึงไนโตรเจนของเชื้อแบคทีเรียมีความสามารถในการตรึงไนโตรเจนในช่วงที่กว้างนั่นคือช่วง 9,001-9,500 nmol C_2H_4 /mg protein/day 1 สายพันธุ์, ช่วง 4,001-4,500 nmol C_2H_4 /mg protein/day 2 สายพันธุ์, ช่วง 3,001-3,500 nmol C_2H_4 /mg protein/day 2 สายพันธุ์, ช่วง 2,001-2,500 nmol C_2H_4 /mg protein/day 1 สายพันธุ์, ช่วง 1,001-1,500 nmol C_2H_4 /mg protein/day 7 สายพันธุ์, ช่วง 501-1,000 nmol C_2H_4 /mg protein/day 15 สายพันธุ์, ช่วง 1-500 nmol C_2H_4 /mg protein/day 194 สายพันธุ์ ผลของการศึกษาลักษณะชีวเคมีพบว่าสามารถจำแนกชนิดของแบคทีเรียตรึงไนโตรเจนอิสระได้ในระดับจีนัส 56 สายพันธุ์ ดังนี้คือ; *Beijerinckia* sp. 16 สายพันธุ์, *Klebsiella* sp. 4 สายพันธุ์, *Azotobacter* sp. 1 สายพันธุ์, *Azomonas* sp. 18 สายพันธุ์ และ *Azospirillum* sp. 17 สายพันธุ์ ผลของการศึกษาการเพิ่มปริมาณ DNA โดยวิธี PCR แบบการใช้ primer *nif* D สามารถแบ่งได้ 48 กลุ่มที่แตกต่างกัน และ primer ERIC พบว่ากลุ่มแบคทีเรียตรึงไนโตรเจนอิสระมีความหลากหลายอยู่สูงมาก และไม่พบว่ามีความสัมพันธ์แบบเจาะจงกับระบบนิเวศแต่ละระบบ

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา .....
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ORAWAN PIYABOON : POLYGENETIC DIVERSITY OF FREE-LIVING NITROGEN FIXING BACTERIA ISOLATED FROM DIVERSED ECOSYSTEMS OF THAILAND
THESIS ADVISOR : PROFESSOR DR. NANTAKORN BOONKERD,
137 PP. ISBN 974–7359–77–4

Polygenetic diversity of free-living nitrogen fixing bacteria isolated from various ecosystem in Thai soils from 3 regions; North, Central and North Eastern were investigated. The soil samples in each part were collected from 11 different types of ecosystems. The bacteria were cultured on N-free media. Free-living nitrogen fixing bacteria were randomly selected for studying as following: 1) morphological and physiological methods, 2) effectiveness of N_2-fixation, 3) biochemical methods and 4) DNA amplification by using primer such as *nif* D and ERIC. The results indicated that bacterial isolates were gram negative. The majority of strains were gram negative rod shape 63.51% and gram negative short-rod shape 36.49%. Effectiveness of N_2-fixation of bacterial isolates were wide ranges in N_2-fixing efficiency that 1 isolate of 9,001-9,500 nmol C_2H_4/mg protein/day, 2 isolates of 4,001-4,500 nmol C_2H_4/mg protein/day, 2 isolates of 3,001-3,500 nmol C_2H_4/mg protein/day, 1 isolate of 2,001-2,500 nmol C_2H_4/mg protein/day, 7 isolates of 1,001-1,500 nmol C_2H_4/mg protein/day, 15 isolates of 501-1,000 nmol C_2H_4/mg protein/day and 194 isolates of 1-500 nmol C_2H_4/mg protein/day. The results of biochemical assay could be used to identify 56 isolates in genera level as following; *Beijerinckia* sp. 16 isolates, *Klebsiella* sp. 4 isolates, *Azotobacter* sp. 1 isolate, *Azomonas* sp. 18 isolates and *Azospirillum* sp. 17 isolates. DNA characterization by using *nif*D-PCR could separate these bacteria into 48 different groups. By using ERIC-PCR found that free-living nitrogen fixing bacteria were high diversity and were not specific to any each ecosystems.

สาขาวิชาเทคโนโลยีชีวภาพ
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ACKNOWLEDGEMENT

This thesis was carried out at School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology during the years 1997-2000. The following funding bodies are acknowledged for financial support : the BRT Research Program Polygenetic diversity of free-living nitrogen fixing bacterial isolated from diversed ecosystem of Thailand.

I would like to expreass my deepest appreciation to Professor Dr. Nantakorn Boonkerd for his kindness, understanding, invaluable supervision, encouragement and financial supports throughout my study.

My appriceation is also to Assistant Professor Dr. Neung Teumroong, Assistant Professor Chokchai Warapu and Dr. Settha Siripin for serving as thesis committee, and their criticisms and valuable suggestion.

Thanks are also exapressed to all staff and member students of School of Biotechnology for their helps in the laboratory with sincerity and frienship.

Finally, I am most grateful to my parents and members of my family for their love, understanding and encouragement.

Orawan Piyaboon

CONTENTS

Page

LIST OF FIGURES

figure Page

figure Page

figure Page

figure Page

figure Page

figure Page

figure Page

figure Page

LIST OF TABLE

LIST OF ABBREVIATIONS

ADP	=	adenosine 5'-diphosphate
AMP	=	adenosine 5'-monophosphate
ARA	=	acetylene reduction activities
ATP	=	adenosine 5'-triphosphate
BNF	=	biological nitrogen fixation
bp.	=	base pair
^{0}C	=	degree celcius
cm	=	centimeter
dATP	=	deoxyadenosine 5' triphosphate
dCTP	=	deoxycytidine 5' triphosphate
DGGE	=	denaturing gradient gel electrophoresis
dGTP	=	deoxyguanosine 5' triphosphate
DNA	=	deoxyribonucleic acid
DMAT	=	dancan multiple test
DTTP	=	deoxythymine 5' triphosphate
EDTA	=	ethylene diamine teraacetic acid
c. g.	=	for example
ERIC	=	enterobacterial repetitive intergenic consensus
et al.	=	et alia (and others)
g	=	gram
h	=	hour
kDa	=	kilodalton
l	=	liter
M	=	molar
ml	=	millilitre
mM	=	millimolar
mm	=	millimeter
mg	=	milligram

LIST OF ABBREVIATIONS (CONTINUED)

mg	=	milligram
min	=	minute
ml	=	millilitre
N	=	normal
NAD	=	nicotinamide adenine dinucleotide
NADH	=	neduced NAD
nm	=	nanometer
nmol	=	nanomole
OD	=	optical density
pmol	=	picomole
REP	=	repetitive extragenic palindronic
RNAseA	=	ribonuclease A
RNA	=	ribonucleic acid
rDNA	=	ribosomal DNA
rRNA	=	ribosomal RNA
rpm	=	revolution per minute
SDS	=	sodium dodecyl surfate
TGGE	=	temperature gradient gel electraphoresis
UV	=	ultraviolet
vol/vol	=	volume per volume

CHAPTER I

INTRODUCTION

Biological Nitrogen Fixation (BNF)

The growth and yield of agricultural crops depend, among other things, on the availability of nitrogen in the soil. It has been estimated that the need for nitrogen in agriculture will be doubled by the year of 2000 due to the increasing global population (Keeney, 1982). Although dinitrogen (N_2) are abundant (78%) in the air, they are not available for plants, only combined inorganic nitrogen can be taken up, so called inorganic fertilizers which are produced from factory by chemical process that require fossil energy. In natural condition molecular dinitrogen in the air enters the organic nitrogen pools in the biosphere through a certain group of prokaryotes which contain nitrogenase enzyme, a complex that fixes N_2 by the expense of ATP, the chemical energy in living organisms, so called biological nitrogen fixing (BNF). The BNF equation was summerized as below (Sprent et al., 1990).

$$N_2 + 8\ \text{ferridoxin}^- + 8H^+ + 16MgATP_2^- + 18H_2O \longrightarrow 2NH_4 + 2OH^- + 8\ \text{ferridoxin} + 16MgADP_4^- + 16H_2PO_4^- + H_2$$

Due to the energy crisis, the price of chemical fertilizer and the risk of environmental pollution are increasing with each passing days, therefore alternative approaches are of great practical interest. One such approach is to use biological nitrogen fixing, a process amplified by a number of diversified genera of bacteria, each genus shows a certain specificity to host plant. There are groups of microorganisms can be devided according to their living form into 2 types: 1) free-living nitrogen fixation such as nonsymbiotic nitrogen fixing bacteria and cyanobacteria (blue green algae) and 2) symbiosis such as rhizobia with leguminous plants (Dobereiner et al., 1975). When compared the efficiency of nitrogen fixation between symbiosis and free-living, it was widely confirmed that symbiosis nitrogen fixer groups perform rather high of nitrogenase activity than free-living group. However, free-living nitrogen fixing bacteria are found to persist more enormous than symbiosis group (Sprent et al., 1990).

Characterics of free-living nitrogen fixing bacteria

The only confirmed free-living nitrogen fixing bacteria belong to the kingdoms eubacteria and archaebacteria. Table1 lists of free-living nitrogen fixing bacterial species, currently known to fix nitrogen. Many are heterotrophic, needing a supply of reduced carbon (e.g. *Azotobacter* sp., *Azospirillum* sp.). Others are autotrophic, that can reduce carbon dioxide. Most (e.g. Rhodospirillaceae) use light energy to do this, by a process which, unlike photosynthesis in cyanobacteria and higher plant, does not evolve oxygen. In addition to the genera listed in table 1 the following thermophilic genera of archebacteria are known to have nitrogen-fixing species: *Methanococcus* sp.. Nitrogen fixation generally occurs under anaerobic, aerobic or micro- aerobic condition. Free-living nitrogen fixing bacteria can be separated into 3 groups by mean of oxygen demand,

1. Aerobic nitrogen fixing bacteria such as family *Azotobacteraceae.*
2. Micro-aerobic nitrogen fixing bacteria such as family *Bacillaceae*, *Enterobacteriaceae* and *Spirillaceae*
3. Anaerobic nitrogen fixing bacteria such as family *Bacillaceae* specific in genera *Desulfovibrio* sp. and *Desulfotomaculum* sp..

Table 1. Diversity of free-living nitrogen fixing bacteria (Dalton and Pastgate, 1980).

Family	Genus	Species
Acetobacteriaceae	*Acetobacter*	*diazotrophicus*
Azotobacteraceae	*Azotobacter*	*beijerinckii*
		chroococcum
		paspali
		vinelandii
	Azomonas	*insignis*
		macrocytogenes
	Azotococcus	*agilis*
	Beijerinckia	*indica*
		fluminesis
		derxii
	Derxia	*gummosa*
	Xanthobacter	*autotrophicus*
		flavus
Bacillaceae	*Bacillus*	*macerans*
		polymyxa
	Clostridium	*pasteurianum*
		saccharobutyricum
		acetobutyricum
		beijerinckia
		tyrobutyricum
		acetobutylifelsin
		lactoacetophilum
		madisoriii
		pectinovorum
		tetanomorphum
		pytyricum
	Desulfotomaclum	*orientis*

Table 1. Continued

Family	Genus	Species
		ruminis
Enterobacteriaceae	*Klebsiella*	*pneumoniae*
		aerogenes
	Enterobacter	*aerogenes*
		cloacae
		agglomerans
	Erwinia	*herbicola*
	Citrobacter	*freundii*
	Escherichia	*intermedius*
Corynebacteriaceae	*Arthrobacter*	*fluorescens*
Rhodospirillaceae	*Rhodomicrobium*	*vannielii*
	Rhodopseudomonas	*palustris*
		capsulata
		gelatinosa
		spheroides
	Rhodospirillum	*rubrum*
Chromatiaceae	*Amoebobacter*	*roseus*
	Chromatium	*minus*
		minutissimum
		gracile
		vinosum
		warmingii
	Ectothiorhodospira	*shaposhnikovii*
	Thiocapsa	*fennigii*
		roseopercicina
	Thiocystis	*violacea*
Chlorobacteriaceae	*Chlorobium*	*thiosulfatophilum*
	Pelodictyon	*luteulum*

Table 1. Continued

Family	Genus	Species
Methanomonadaceae	*Methylosinus*	*richosporium*
		sporium
	Methylococcus	*capsulatus*
Thiobacteriaceae	*Thiobacillus*	*ferro-oxidans*
Pseudomonadaceae	*Pseudomonas*	*diazotrophic*
		pseudoflava
		saccharophila
		stutzerl
Spirillaceae	*Azospirillum*	*lipoferum*
		brasilense
		amazonense
	Aquaspirillum	*peregrinum*
		fasciculus
	Herbaspirillum	*campylobacter*
Vibrionaceae	*Vibrio*	*diazotrophicus*
		natriegens
Uncertain family	*Desulfovibrio*	*desulfuricans*
		vulgaris
		gigas
	Alcaligenes	*latus*
Methanobacteriaceae	*Methanococcus*	*hermolithotrophicus*
		voltae

The nitrogenase complexes

The biochemical study of nitrogenase has begun before 1960 (Ladha, 1982). The ability to fix atmospheric N_2 of diazotroph is involved with the nitrogenase and the genetic expression of nitrogen-fixation *(nif)* genes. The nitrogenase consists of two components. Both components form aggregates and can be shown to consist of subunits. By the study from free-living nitrogen fixing

bacteria the nitrogenase complex is composed of two components (kpI and kpII) required for N_2-fixing activity. The component I (kpI) a dinitrogenase or molybdoprotein (MoFe protein) consists of two *a*- and *b*- subunits (a_2 b_2). The *a*- and *b*- subunits are coded by *nifD* and *nifK,* respectively. The component II (kpII), a dinitrogenase reductase or iron protein (Fe protein), consists of two identical subunits coded by *nifH.* These protein components are irreversibly inactivated by oxygen. A cofactor (FeMOCo) containing Mo, Fe and S, presumed to carry the active of nitrogenase and can be separated from the MoFe protein (Shah and Brill, 1977). Earlier reports that *Azotobacter* sp. could occasionally fix nitrogen in the absence of molybdenum have now been fully substantiated and two alter forms of nitrogenase have so far been found. The most widely studied of these contains vanadium in place of molybdenum while the other appears to lack both Mo and V. In the presence of Mo the 'conventional' nitrogenase always appears to be formed. These alter native systems have been reviewed by Joerger and Bishop (1988). Both a reductant and ATP are required for enzyme activity; approximately 15 mole of ATP are consumed per mole of dinitrogen reduced. In view of this high-energy requirement and the extreme oxygen sensitivity of the protein components it is not surprising that nitrogenase synthesis is tightly controlled. Table 2 summary of *nif* genes found in free-living nitrogen fixing bacteria, in the order in which they occur in the chromosome, and their possible function.

Table 2. The products and functions of *nif* genes of free-living nitrogen fixing bacteria (Arnold et *a*l., 1988).

Gene	Molecular Mass (KD)	Function
J	120	Electron transport: pyruvate flavodoxin oxidoreductase
H	35	Dinitrogenase reductase (Fe protein, component II)
D	56	*a*- Subunit of dinitrogenase (MoFe protein, component I)
K	60	*b*- Subunits of dinitrogenase (MoFe protein, component I)
T	-	unknown
Y	24	Maturation of component I
E	40	Synthesis of FeMoco
N	50	Synthesis of FeMoco
X	18	unknown
Y	25	Maturation of component I
S	45	Maturation of component I or component II
V	42	Synthesis of FeMoco:homocitrate synthase
W	-	unknown
Z	15-17	unknown
M	28	Processing of component II
F	20	Electron transport:flavodoxin
L	50	*nif*-specific repression
A	60	*nif*-specific activation
B	49	Synthesis of FeMoco
Q	-	processing of Mo

Importance of nitrogen fixation

Forest soils are ecosystems in which nitrogen availabilary limited. Nitrogen may be available through decomposition of organic material or fixation of molecular nitrogen. The nitrogen contents of coniferous plant litter and the loss of nitrogen through mineralization contribute to its limited availability. Relating experimentally determined indigenous nitrogenase activitied and nitrogen accumulation to the expected activities of know free-living and associative nitrogen fixers in forest systems leaves portions of the N input unaccounted for (Widmer et al., 1999). Agriculture in the tropics might be expected to be more dependent of N fertilisers than that in temperate regions, because heavy rains and more rapid organic matter decomposition lead to leaching and rapid losses of the N fertilisers applied. In addition to ground water pollution other problems due to high N fertiliser applications occur in the tropics. High N fertiliser prices especially in Brazil where they are not subsided can make agriculture unprofitable. Due to this plant genotypes have been selected for high yields with low or no N fertilisers. It can be seen that among all countries in the world. Soil microorganisms mediate many processes that are essential to the agricultural productivity of soil. These processes include recycling of plant nutrients, maintenance of soil structure, degradation of agrochemicals and pollutants and the control of plant and animal pests. Although the relationships between soil microbial diversity and the functioning and sustainability (or stability) of agricultural ecosystems are unclear examples which show that diversity of soil biota is important in key functions of agroecosystems have been documented. On a global scale, it is generally agreed that free-living bacteria do not make a major contribution to nitrogen in put in agricultural system. It has been estimated that up to 33 kg N ha^{-1} may be fixed by clostridia in flooded meadow-bog soils and rice fields in Russia. The predominant species of *Clostridium* sp. found in soils varies with latitude, as does the nitrogen fixing capacity. In northern regions the breakdown of organic carbon is slower than further south and the C: N ratio is higher. Under these conditions bacteria fix more nitrogen per unit of glucose. This reflects the fundamental requirement of non-photosynthetic free-living bacteria – a plentiful supply of carbon, which clearly restricts their range. Soils on which crop plants flourish generally need adequate aeration. Rice is exception in that it can survive flooding by producing more aerenchyma and by transporting oxygen from shoot to root. Flooded rice fields (which comprise about 75% of rice cultivation) may represent the most significant input by free-living nitrogen-fixing organisms into

agriculture. Keeney and Sahrawat (1986) suggested that significant nitrogen fixation may occur in the flood water (aerobic bacteria), reduced soil (anaerobic bacteria) and the rhizosphere which is partially oxidized and may support nitrogen-fixing organisms both on or in the root. Hill and Patriquin (1988) report that the addition of an aerobic nitrogen-fixing crude culture from sugar cane litter to wheat straw with or without the indigenous wheat microflora resulted in gains of up to 6.6 mg N g^{-1}. They calculated this as equivalent to 33 kg N-fixed ha^{-1} for a 5 t ha^{-1} straw crop. Not only does the method show potential in reducting fertilizer nitrogen requirement for later crops, but the enhanced rate of nitrogen fixation also accelerates straw breakdown. Large numbers of *Azotobacter* sp. and *Clostridium* sp. are found in Egyptian soil despite a usually low soil organic content. When soil organic matter rises, normally as a result of crop residues, the population of nitrogen species -principally *Azotobacter* sp. rises rapidly, and by inference, enhances soil nitrogen.

Environmental factor effecting diversity of free-living nitrogen fixing bacteria

The ability of free-living nitrogen fixing bacteria to actually fix N_2 in the field is strongly influenced by the prevailing environmental condition. Although the tropics contain some of most productive environments in the word, they also contain their fair share of hostile environments. The main environmental stresses which occur in the tropics can be divided into predominantly physical factor (temperature, moisture) and into chemical factors which include both toxic effects acidity, aluminium and nutrient deficiencies. Such environmental stresses can act several different levels. They may reduce the survival or rate of growth of microorgannisms in free-living state. A single environmental stress may, of course, affect on or all of these process.

A. Physical factors

Temperature. In part of the tropic the surface soil temperature can occasionlly reach 60-70 °C and temperature above 50°C can be found at 5 cm depth (Dudeja and Khurana, 1989). Most heterotripic free-living nitrogen fixing bacteria are not highly resistant to desiccation. Survival of bacteria in soil at high temperature appears to be improved by the presence of clay particles and soil organic matter, but many of the soil where high temperature are experienced sandy. In general, bacteria are loss tolerant of high temperature in moist than in dry soil. Nitrogenase from *Azotobacter* sp. can reduce acetylene to increasing ethylene in range 10-30 °C

but it is appropriate temperature about 20-25 °C. *Azotobacter* sp. is grow and increase N_2-fixer in range 20-30 °C which the similar strain as *Azomonas* sp. and *Beijerinckia* sp.. For *Azosprillum* sp. is grow and perform N_2 – fixing ability fixing N_2 between 20-40 °C (Dobereiner, 1983).

Drought. Bacterial strains which survive under greater water stress are those which retain less water within the cells (Bushby and Marshall, 1977). Rate of N_2-fixation are more sensitive to reduction in soil water content than other processes such as photosynthesis, transpiration or leaf growth rates (Sinclair et al., 1987). Even in species which are grown in arid regions and are considered to be tolerant of drought, slight changes in the plant water potential cause a marked reduction in both the rate of N_2-fixation to the shoot (Ro and Venkateswarlu, 1987).

Saline and sodic soils (pH). Saline and sodic soil effect about population and distribution in *Azotobacter* sp.. pH is usually above 4.9-9 but pH for N_2-fixation is 5.5-7.2. Low pH of soil or sodic soil inhibit growth and N_2-fixation of *Azotobacter* sp. more than high pH soil. *Azosprillum halopraeferens* isolated from the rhizosphere of plants growing in a highly-saline environment have some tolerance to salt (Singleton et al., 1982).

Oxygen. Aerobic diazotrophs must regulate the oxygen supply both to provide ATP and protect nitrogenase against oxygen damage at the same time. Diazotrophs have developed many strategies for limiting oxygen access to nitrogenase, from growth in micro-aerobic conditions to morphological changes, such as legume nodules, heterocysts in cyanobacteria, vesicles in non legumes and the variable oxygen barrier in nodule cortex cells. Perhaps the best understood of all protection mechanisms are the two proposed: respiratory and conformational protection in *Azotobacter* sp., the most aero-tolerant of all diazotrophs. First reported H_2 production by soybean nodules. However, having failed to find H_2 production by N_2 production by N_2-fixing *Azotobacter vinelandii* suggested in might be an *in vitro* artefact of isolated nitrogenase activity. These doubts were removed when it was shown that both symbiotic and asymbiotic systems produced H_2-uptake hydrogenases of aerobic diazotrophs are dimeric ($\alpha\beta$) nickel-iron, membrane-bound proteins which catalyse H_2 oxidation via the respiratory chain to produce ATP *in vitro*. In pure cultures the H_2 derives solely from nitrogenase activity and may benefit the organism by providing ATP, contributing to respiratory protection and removing a potential inhibitor of N_2 fixation (H_2) from the vicinity of the nitrogenase site (Yates et al., 1997).

In the case of *Azospirillum lipoferum* and *A. brasilense* , oxygen protection is possible through clustering or clumping of the bacteria. These clusters can establish steep oxygen gradients. *Azospirillum* sp. has some respiratory protection of its nitrogenase and is capable of fixing nitrogen optimally at 0.5% oxygen in broth culture. It may have greater protection from oxygen when in association with the root duc to colonisation pattern (Dobereiner, 1983).

Moisture. Nitrogen fixing bacteria require soil moisture to 60-70 % of soil water holding capacity because bacteria are using C- source. Weier (1980) reported that the high soil moisture effected high quantity of N_2-fixing. Moisture or soil water are important to move nitrogen from rhizospere of plants (Watanabe, 1981).

B. Chemical factors

Toxicities. Recent studies have shown that pesticides and herbicides can decrease microbial respiration, biomass, and diversity. Given the large proportion of tropical soil which are acid, problems associated with low pH are of wide spread importance. These problems can arise from to cases. The first is the simple problem of survival in a medium of low pH. The second category of problem result from other chemical changes in soil causes by high acidity, particularly the large amounts of aluminium and also iron and manganese that may come into solution, the corresponding decreases in availability of phoshorus and molybdenum and the lack of calcium in most acid soils (Giller et al, 1991).

C. Effect of nutrient

Several of the nutrients essential for growth of plants or bacteria play specific roles in N_2-fixing. Deficiencies in there nutrients, or other nutrients essential for the growth of bacteria or plants, can cause reduction in the numbers and size formed and in the amount of N_2-fixed.

Nitrogen. A plentiful supply of ammonium prevents nitrogen fixation in cultures of diazotrophs and also, by inference, in the natural habitats occupied by these bacteria. In addition to ammonium, other compounds such as amino acids or nitrate can prevent nitrogen fixation, though their effects are less well documented than those of ammonium. As with most other aspects of nitrogen fixation, the mechanisms by which ammonium inhibits nitrogen fixation are best understood in the group of prokaryotes such as Proteobacteria, formerly known as purple bacteria. In species of *Azospirillum* sp., *Rhodospirillum* sp., *Rhodobacter* sp. and one species of *Azotobacter* sp., *A. chroococcum*, addition of ammonium at concentrations of about 1-10 mM to

cultures actively fixing nitrogen decreases nitrogenase activity almost immediately to 10% or less of initial activity. Also in these organisms, no new nitrogenase was synthesized. In the other genera, addition of ammonium generally leads to a gradual decline in nitrogenase activity, by about half for each generation of new growth. This reflects a lack of synthesis of any new nitrogenase, but not inhibition of nitrogenase already present at the time of ammonium addition. Thus, in any particular diazotroph, ammonium may inhibit both nitrogenase activity and synthesis or only prevent nitrogenase synthesis. (Rudnick et al., 1997).

Other nutrients. Fe, P, Mo, Co and K which are essential component of growth as well as necessary for synthesizing the nitrogenase enzyme (Heselkorn and Buikema, 1992).

D. Biological factors

Growth and survival of free-living N_2-fixing bacteria will be influenced by competition and antagonism from other organisms. Some microorganisms were including fungi and other bacteria (Giller et al., 1991). *Azotobacter* sp. can active for phosphobacteria and *Azosprillum brasilense* helps *Cellulomonas gelida* to hydrolysis cellulose. Because *C. gelida* is received nitrogen and amino acid or vitamin (Sprent et al., 1990).

Characterization of free-living nitrogen fixing bacteria

Morphological, physiological and immunological methods

Morphology of free-living nitrogen fixing bacteria study shape, colony, slime, gram staining of cell and shape of cell (Dobereiner, 1971). The study about genetic diversity by different isolation media and conditions are required to obtain these bacteria (Ueda et al., 1995). Because of their diversities, different isolation media and conditions are required to obtain these bacteria from the same source. It is most interesting to compare associative N_2 -fixer such as *Klebsiella oxytoca* with their relative which are free-living N_2-fixer (*Klebsiella pneumoniae*) or associative N_2-fixer in different geography i.e. temperate region (Japan) and tropical region (Philippines, Thailand); especially their specific differences in biochemical characters. For the physiology and biochemistry of N_2 -fixation of these organisms has been studied mainly to determine the oxygen tolerance of their nitrogenase activity. However so far very little has been known about the utilization of carbon and energy source for N_2 -fixation. Most examinations have been carried out with glucose as the carbon source. Other method for the identification these

species is based on immunological techniques by FA Technique that it use to examine and identify of bacteria (Ladha, 1982).

Determination of base composition of deoxyribonucleic acid (DNA)

A new approach to the study the diversity of free-living nitrogen fixing bacteria is to analysis DNA pattern. First method is G+C content determination that one of is important parameters to distinguish free-living nitrogen fixing bacteria from other microorganisms. If significant differences in DNA base composition are to occur between the bacterial groups, this will be the evidence for the existence of more than one genus and the similarity within the bacteria groups indicated that the strains can possible, but not necessarity, all belong to the same genus. Choonhahirum (1986) supports that the mol % G+C volumes of bacterial strain R15 and R17 is similar to *Klebsiella oxytoca* 1301 but significantly different from standard strains of *Azospirillum* spp. and *Pseudomonas* spp..

Plasmid pattern and restriction cut

Plasmid are small circular extrachromosomal DNA found in some bacterial cell. In general, they carry some genes that controled the synthesis of specific enzyme essentially for bacterial or some genes conferring resistance to antibiotics or drugs. In some cases, they also have some genes controling toxins production. By conjugation, plasmid can be transferred into new bacteria and then transfer their propertier to that host. These genetic elements are sometimes nonessential for growth so that under many conditions they can be lost or gained without harm to the cell (Brock, 1974). Robson et al. (1995) show that the plasmids are detected in recent isolates of *Azotobacter chroococcum* from local soils exhibit variability in number and size. For plasmid pattern and the enzyme restriction cut pattern of the bacterial chromosomal DNA indicate that it is related bacteria should result in similar plasmid pattern. Choonhahirum (1986) study about plasmid detection in N_2 –fixing bacteria that the results show bacterial strain R15 and R17 harbor a plasmid of molecular weight large than 33 Mdal where *Azospilillum lipoferum* 34H, A. *lipoferum* FS, *Pseudomonas* sp. H8 and *Pseudomonas* sp. KLH76 harbor a plasmid of molelular weight very close to the molecular weight of chromosal DNA. The restriction cut plasmid profile of bacteria strain R15 and R17 are similar to each other but the plasmid profile of bacterial strain R25 is similar to *A. lipoferum* 34H and A. *lipoferum* FS which are different from, *Pseudomonas*

sp. H8 and *Pseudomonas* sp. KLH76. No plasmid is detected in *Klebsiella oxytoca* and thus it may be considered as plasmidless.

Molecular biological method

PCR amplification

The method developed by PCR in order to determine species diversity and composition, using rRNA (or ribosomal DNA) isolated directly from nature has opened a window into the world of unculturable bacteria. Although this method has the disadvantage that organisms whose genes have been isolated by the method cannot be studied for any other traits (Pace et al., 1986). This report extends the method by applying it to a functionally important gene, *nif* gene as conventional nitrogenase, the enzyme that catalyzes biological dinitrogen reduction to ammonium, is composed of two highly conserved proteins: the iron (Fe) protein (encoded by the *nifH* gene) and the molybdenum iron (MoFe) protein (encoded by the *nifDK* genes). The nitrogenase enzyme is present in diverse lineages of prokaryotes and is generally believed to be ancient. *nif* H gene amplified from oceanic water showed that the open ocean contain more diverse diazotrophic microbial population and most divers habitats for nitrogen fixers than previously observed by classical microbiologal technique (Zehr et al., 1998). Ueda et al. (1995) have also taken a similar approach to analyse the diversity of nitrogen - fixing organism in the rhizosphere of rice roots, using universal *nif D* primer. The development of randomly amplified polymorphic DNA, ERIC (Enterobacterial Repetitive Intergenic Consensus) and REP (Repetitive Extragenic Palindronic) markers provided a new tool for investigate genetic polymorphismes in different organisms (Jayarao et al., 1992).

Temperature gradient gel electrophoresis

Auother approach to the study of the diversity of natural microbial communities is analysis of PCR products generated with primers homologous to relatively conserved regions in the genome via denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE). These approaches allow separation of DNA molecules that differ by single bases and hence have the potential to provide information about variations in target genes in bacterial populations in natural systems. Moreover, by adjusting the primers used for amplification, both major and minor constituents of microbial communities they can be characterized (Heuer et al., 1997). Temperature gradient gel electrophoresis performed with 16S

ribosomal DNA (rDNA) amplicons has already been used to study the genetic diversity of microbial communities in environments such as the potato rhizosphere (Heuer et al., 1997) and also to detect sequence heterogeneities in single genomes. PCR-DGGE has also been used similarly in a range of different environments (Rolleke et al., 1997). The discrimination of different strains by denaturing gradient gel electrophoresis of the [NiFe] hydrogenase plays an important role in the energy metabolism of *Desulfovibrio* sp. (Wawer et al., 1997).

Impact of free-living nitrogen fixing bacteria

Free-living nitrogen fixing bacteria are a number of diversified genera of nitrogen fixing microoganism that mostly found in the soils. The better understanding of this nitrogen fixation process might help to improve the nitrogen fixing ability of the nitrogen fixers by means of manipulation. Although it is impossible now to replace absolutely conventional nitrogen fertilization with biological nitrogen fixation in growing main crops but upon proper improving system of free-living nitrogen fixing bacteria grain yield may soon become less depend on the industrially produced nitrogen fertilizer. Increasing human population put a great pressure on land requirement for housing and food production resulted in large part of forest and plant resources are damaged. This lead to the change in environment and ecosystem which is directly effect change in microorganisms communities including free-living nitrogen fixing bacteria. Thus, it is important to find the way for protection the nitrogen fixing bacteria from risk for maintaining sustainably agriculture and forestry in the future.

Objective of the thesis

The objective of this study was to investigate biodiversity of free-living nitrogen fixing bacteria isolated in Thailand with regards to diversification in genetic level and in conjunction with population changes in diversed ecosystems. This might be able to bring about the management for proper utilization of free-living nitrogen fixing bacteria for increasing soil fertility in sustainable agriculture and forestry.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

1. Free-living nitrogen fixing bacterial strains

The bacterial cultures used as free-living nitrogeen fixing bacterial reference strains were as follow: *Azospirillum brasilense* Sp7, *A.lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 (obtained from Dr. Zulkifli H. Shamsuddin from Universiti Putra Malaysia). *Beijeranckia* sp. and *Azotobacter* sp. were obtained from Dr. Settha Siripin, Department of crop Sciences, Faculty of Agriculture, Mae-jo University and *Escherichia coli* HB101.

The bacterial isolates were isolated from the various soil samples in Thailand. Site selection was conducted in 3 regions ; North, Northeast and Central. In each region, soil samples were collected from highest elevation as on the top of the mountain, in the middle and at foot hill of mountain. Soil samples were also collected from the flat area of agricultural practice as field crop cultivation, rice cultivation, rice in rotation with other crops and uncultivated area. The period of soil sample was done every two month since 1997 to 1999. Bacteria were isolated by Dr. Settha Siripin from Department of Agronomy, Faculty of Agricultural Production, Mae-jo University.

2. Culture media and cultivation

2.1 Nitrogen free Agar (Dobereiner et.al., 1972)

Dipotassium hydrogen phosphate, K_2HPO_4	0.1	g
Potassium dihydrogen phosphate, KH_2PO_4	0.4	g
Calcium chloride, $CaCl_2$	0.02	g
Magnesium sulphate, $MgSO_4$	0.2	g
Sodium chloride, NaCl	0.1	g
Ferric chloride, $FeCl_3$	0.01	g
Sodium molybdate dihydrate, Na_2MoO_4	0.002	g
Glucose	10	g
Distilled water	1	l
pH 6.8		

Modified nitrogen free medium with various C-sources amount of sugars was used same the source as in nitrogen free agar medium.

2.2 Malate agar (Dobereiner et al., 1972)

DL-malic acid	5	g
Potassium dihydrogen phosphate, KH_2PO_4	0.4	g
Magnesium sulphate, $MgSO_4.7H_2O$	0.2	g
Sodium chloride, NaCl	0.1	g
Calcium chloride, $CaCl_2$	0.02	g
Sodium molybdate, Na_2MoO_4	0.002	g
Potassium hydroxide, KOH	4	g
Distilled water	1	l
Bromthymol blue (0.5%) in ethanol	2	ml
Agar	15	g
pH 6.8		

2.3 Methyl Red-Voges-Proskauer broth (Krieg, 1981)

Peptone	7	g
Dipotassium hydrogen phosphate, K_2HPO_4	5	g
Glucose	5	g
Distilled water	1	l
pH 6.8		

2.4 Potato dextrose agar

Potato	200	g
Dextrose	20	g
Agar	15	g
Distilled water	1	l
pH 6.8		

2.5 Ammonium Salt Sugar

Diammonium hydrogen phosphate	1	g
Potassium chloride	0.2	g
Magnesium sulfate	0.2	g

Yeast extract	0.2	g
Agar	20	g
Distilled water	1	l
Bromcresal purple 0.2% in water	4	ml
C-source		
- 1% Arabinose in water	100	ml
- 1% Rhamnose in water	100	ml
- 1% Glucose in water	100	ml
- 1% Mannitol in water	100	ml
- 1% DL-Malic acid in water	100	ml

2.6 Luria broth

Tryptone	10	g
Yeast extract	5	g
Sodium chloride	5	g
Distilled water	1	l

pH 7

3. Chemicals

All chemicals used were laboratory grade, or otherwise specified

3.1 For acetylene reduction activity (ARA) measurement

3.1.1 Gases

Compressed air :From the Military Science Department

Hydrogen (H_2) : From the Military Science Department

Ethylene standard (C_2H_2) :From Thai Industrial Gases Limited

Nitrogen (N_2 OFN) : From Thai Industrial Gases Limited

Acetylene (C_2H_2) : From Calcium carbide + H_2O

3.1.2 Auto System XL Gas Chromatograph from Perkin Elemer, inc. USA.

3.1.3 Column packed with porapakN From Perkin Elmer, inc. USA.

3.2 Reagents for determination of protein by Lowry methos (Robert, 1988)

Sodium chloride, 0.85% NaCl in distilled water

Bovine serum albumin in 0.85% sodium chloride

Sodium hydroxide (NaOH), 1 N in distilled water

Potassium tartrate, 2% in distilled water

Copper sulfate ($CuSO_4$), 1% in distilled water

Sodium carbonate (Na_2CO_3), 2% in distilled water

Folin Ciocalteau Phenol Reagent, 1 N in distilled water

3.3 Reagents for DNA extraction (Schmidt et. al., 1986)

TNE buffer: 10mM Tris, 1 mM EDTA, 0.1M NaCl in distilled water (pH 8)

Lysozyme solution: 2 mg/ml lysozyme in 25 mM Tris, 12.5 mM EDTA, 12.5% sucrose in distilled water(pH 8)

Lysing solution: 10% SDS in distilled water

Phenol-chloroform-isoamyl alcohol, 25: 24:1 (v:v)

Chloroform-isoamyl alcohol, 24:1 (v:v)

Ethanol, 99%and 70%

Sodium acetate, 3N

TE buffer: 10 mM Tris, 1mM EDTA in distilled water (pH 8)

RNase A, 100 μg/ml in 0.1 M sodium acetate (pH5.6)

3.4 Reagent for PCR amplification

Taq DNA polymerase in storage buffer B (Promega), size:500U; concentration: 5u/μl

Mangasium chloride solution, 25 mM

10x buffer Deoxynucleoside triphosphate, 2.5 mM

3.5 Reagent for agarose gel electrophoresis

DNA marker: 1 kb Ladder DNA, 100 bp. Ladder DNA(Promega and GibcoBRL)

Staining solution: 0.5 μg/ml ethidium bromide in distilled water

Tracking dye: 0.25% bromocresol purple in 50% glycerol 0.05 M Tris-acetate (pH 7.9)

TBE buffer: 1 mol/l Tris, 0.83 mol/l Boric acid and 10 mol/l EDTA (pH 8)

3.6 Reagent for Gram Staining

Iodine solution: 0.3 mg/ml iodine in distilled water, 0.6 mg/ml potassium

iodide in distillled water

Ammonium oxalate crystal violet solution: 0.1 g/ml Crystal violet in 95% ethanol, 1% ammoniumoxilate in distilled water

Safranin solution: 2.5% safranin in 95% ethanol

Ethanol, 95%

3.7 Reagent for Voges-Proskauer test solution (Krieg, 1984)

Solution A: Alpha-napthol solution ; 5% alpha-napthol in absolute ethanol

Solution B: KOH (40% solution) ; 40% potassium hydoxide in distilled water

METHODS

1. Morphological and physiological study of free-living nitrogen fixing bacteria

1.1 Bacterial morphological study

The isolates from each area were randomly selected on the basis of distinction of selected Gram staining and colony formation

1.2 Detection for effectiveness of N_2 - fixation

1.2.1 Determination of Nitrogenase activity

Nitrogenase activity was determined by acetylene reduction assay (ARA). The bacterial culture was carried out in 20 ml-tube containing 5 ml liquid medium and the assay was done on 1 set of 3 replications. The bacteria were cultured for 7 days at 30 ^{o}C then they were used for ARA . ARA was done by added 10% acetylene which was adjusted by removing air and replacing with equal volume of acetylene. At intervals of 24 hours 1 ml of the gaseous phase was injected into a gas chromatograph under the following conditions. Column packing = Parapak N, column temperature = 110^{o}C and detection temperature 280^{o}C. The assay was performed after cultivation of free-living nitrogen fixing bacteria for 7 days. The standard pure ethylene gas (99.9%) was diluted by air and injected at increasing volume as standard before every assay.

1.2.2 Protein determination by Lowry method (Robert, 1989)

The cell pellets were collected by centrifugation at 12,000 rpm for 10 min at 4^{o}C. The pellet was suspended twice with 1 ml of 0.85% NaCl. After centrifugation at 12,000 rpm for 5 min at 4^{o}C the supernatant was discarded. The pellet was suspended in 0.2 ml of 0.85%

NaCl by pipetting up and down and transferred into a new test tube. The supernatant was added with 0.7 ml of sterlied water and 0.1 ml of 1N NaOH and then followed with 5 ml solution C (2% of potassium-tartrate, 1% of $CuSO_4$ and 2% of Na_2CO_3) to each of sample tube. Each sample was throughly mixed before incubation at room temperature for 10 min. After which 0.5 ml of 1N Folin Ciocalteau phenol reagent were added and mixed, incubated at room temperature for 30 min. To determine the protein concentration, the reaction in each tube was read at wavelength of 660 nm in comparision with BSA standard at concentration of the 100 μg/ml, 50 μg/ml, 25 μ g/ml, 12.50 μg/ml and 6.25 μg/ml.

1.3 Biochemical assay

1.3.1 Voges-Proskauer reaction (Krieg, 1981)

The tube of methyl red- voges- proskauer was used in broth culture before incubated at 30°C for 48 hours. Culture of 1 ml of culture was added to another tube containing 0.6 ml of 5% (w/v) α-naphthol (dissolved in absolute ethanol), mixed thoroughly, then 0.2 ml of 40% aqueous KOH was added. After mixing well, the tube was incubated in slant position to increase the surface area of medium. Strong red color began at the surface of medium after 15 or 60 min of incubation.

1.3.2 Potato dextrose agar

Bacterial culture was streaked on potato detrose agar and incubated at 30°C for 48 hours. The colour of colonies was recorded for further identification.

1.3.2 Utilization of various carbon sources

Bacterial culture was streaked on ammonium salt sugar and incubated for 2 days at 30°C. For positive reaction changes of green to yellow colour were detected.

2. DNA extraction and PCR analysis

2.1 Genomic DNA extraction

Cells were grown in 1.5 ml of modified nitrogen free medium (added 0.05% yeast extract) for 1-2 days at 30°C and cell pellets were harvesteded by centrifugation at 12,000 rpm for 5 min at 4°C. Cell pellet was resuspended twice with 1 ml TNE (10mM Tris [pH8.], 1mM EDTA [pH8], and 0.1 M NaCl). After centrifugation at 12,000 rpm for 5 min at 4°C the supernatant was discarded. The pellet was then suspended with 200 μl lysozyme solution (2 mg/ml lysozyme in 25 mM Tris [pH8], 12.5 mM EDTA [pH8] and 12.5% sucrose), incubated for 30 min at 37°C

before 250 μl lysing solution (10% SDS solution) was added, vortexed and incubated for 2 hours at 37^{o}C. The solution was transferred to another microcentrifuge tube and repeated the step of phenol extraction twice. DNA from aqueous phase was precipitated for overnight (-20^{o}C) with 45 μl of 3M sodium acetate and 900 μl of 99% ethanol. The DNA pellet was washed again with 70% ethanol before dried under vacuum and resuspended with 30 μl of TE buffer (10 mM Tris [pH8], 1mM EDTA [pH8] with 1/10 RnaseA) then incubated at 37^{o}C for 30 min for and collected in 4^{o}C for futher uses. (Schmidt et al., 1986).

2.2 PCR amplification

Primer 1: 5'- AT (TC) AGGGT(TCGA) CTCTCGTA(GA)AC(TCGA)GCCTT- 3' and primer 2 : 5'-AT(GC) GA(AG)T (AT) C AAC TTC TCCGG-3' were used for the PCR amplification of the *nif*D segments. The region amplified was a relatively variable part of the molecule and thus was a high density of information. The *nif*D gene cluster of *Azotobacter vinelandii* was shown in Figure 1. The *nif*D gene was amplified from 50 ng of DNA template (negative controls : water instead of DNA and positive controls : DNA of reference strains) by using 2.5 U of Taq DNA polymerase (Promega, U.S.A.) which carried out in the total reaction volum of 25 μl, 0.95 pmol and 1.025 pmol of primer 1 and 2 of *nifD*, respectively, 200 μM deoxynucleoside triphosphate, 1.5 mM $MgCl_2$ and 10xPCR buffer. The reaction conditions were as follows: 4 min at 94^{o}C and 3 min at 72^{o}C for the first 1 cycle. 0.5 min at 94^{o}C, 0.5 min at 35^{o}C and 2 min at 60^{o}C for the second 5 cycles. 0.5 min at 35 ^{o}C and 2 min at 60 ^{o}C for the third 30 cycles. Followed by 1 cycle with 3 min at 72 ^{o}C and 2 min at 4 ^{o}C in PCR Sprint Temperature Cycling System. The oligonucleotide primer used were synthesized with an Applied Biosystems Model 380 B DNA synthesizer and kindly provided by J. Lupski (Baylor Collego of Medicine, Houston, Tex). For auother primers, Enterobacterial repetitive intergenic consensus (ERIC) ; ERIC-IR :5'- GTGAATCCCCAGGAGCTTACAT -3'and ERIC-2 : 5'- AAGTAAGTGACTGGGGTGAGCG -3'was used together with 50 ng of genomic DNA as the DNA template (negative controls : water instead of DNA and positive controls : DNA of reference strains). For PCR amplification which carried out in the total reaction volume of 25 μl, 0.95 pmol primer ERIC-IR and 0.80 pmol primer ERIC-2, 200 μM deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10xPCR buffer and 2.5 U of Taq DNA polymerase (Promega, U.S.A.). The amplification condition were as follow: 7 min at 95 ^{o}C for the first 1 cycle, 1 min at 94 ^{o}C, 1 min

at 52 ^{o}C and 8 min at 65 ^{o}C for the second 30 cycles, 16 min at 65 ^{o}C for the third 1 cycle. Amplification was performed in PCR Sprint Temperature Cycling System. After the amplification, 15 μl aliquots of the PCR product were resolved by gel electrophoresis at 80 V/cm in 1.2 % agarose gel (Promega, U.S.A.) stained with the ethidium bromide.

3. Phylogenetic analysis

Dendrogram were construted from the similarrity matrix by the unweighted pair group method with arithmetic mean (UPGMA). In order to test the goodness of fit of cluster analysis, cophenetic value matrices were calculated and compared with the original similarity matrics that were UPGMA clustered by using the NTSYS-pc package (Version 1.8; Exeter software, Setauket, N.Y.).

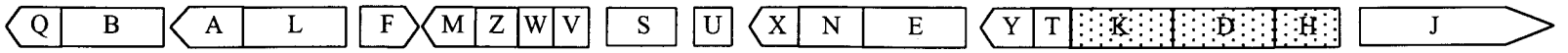

Figure 1. The *nif* gene cluster of *Azotobacter vinelandii*

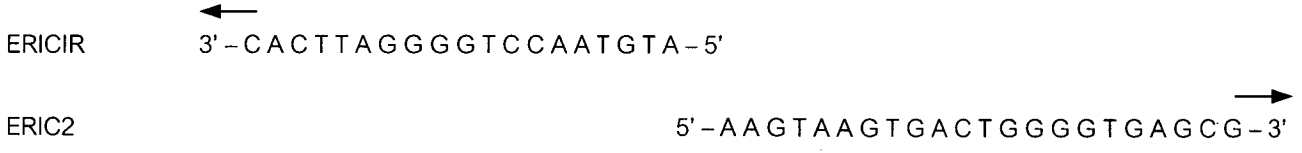

Figure 2. Nucleotide sequence of the two ERIC primer (ERICIR and ERIC2), positioned relative to the ERIC consensus sequence. The arrows denote the direction of taq polymerase extension

CHAPTER III

RESULTS

3.1 Morphological and physiological study of free-living nitrogen fixing bacteria

The free-living nitrogen fixing bacteria were isolated on N-free media by Dr. Settha Siripin from Mae-Jo University. The bacteria were randomly selected for studing as:

3.1.1 Gram staining

For morphological study by gram staining indicated most isolates were gram negative rod shape 63.51% and gram negative short-rod shape 36.49%. The data of all size were summarized in appendix A.

3.1.2 Detection Effectiveness of N_2-fixation

Effectiveness of N_2-fixation was determined by acetylene reduction assay (ARA). Wide range in N_2-fixing efficiency (1-9,500 nmol C_2H_4 /mg protein/day) was detected (Table 7). It was shown that only one isolate had the highest ARA of 9,001-9,500 nmol C_2H_4 /mg protein/day. The other effectiveness were as following; 2 isolates of 4,001-4,500 nmol C_2H_4 /mg protein/day, 2 isolates of 3,001-3,500 nmol C_2H_4/mg protein/day, 1 isolate of 2,001-2,500 nmol C_2H_4 /mg protein/day, 7 isolates of 1,001-1,500 nmol C_2H_4 /mg protein/day and 15 isolates of 501-1,000 nmol C_2H_4 /mg protein/day. However all isolates of 194 isolates could reduce acetylene in minimum range of 1-500 nmol C_2H_4 /mg protein/day. The results obtained from this study was confirmed that the bacteria isolated from soil samples were N_2-fixing bacteria. The data of ARA in each area was summarized in Table 3.

Table 3. N_2-fixation efficiency of free-living nitrogen fixing bacteria isolated from each area

ARA (nmol C_2H_4 /mg protein/day)	No. of isolates
9,001-9,500	1
8,501-9,000	0
8,001-8,500	0
7,501-8,000	0
7,001-7,500	0
6,501-7,000	0
6,001-6,500	0
5,501-6,000	0
5,001-5,500	0
4,501-5,000	0
4,001-4,500	2
3,501-4,000	0
3.001-3,500	2
2,501-3,000	0
2,001-2,500	1
1,501-2,000	0
1,001-1,500	7
501-1,000	15
1-500	194

3.1.3 Biochemical assays

The results obtained form these phenotypic characters, other biochemical and physiological properties of reference strains as shown in Table 4.

Table 4. Distinction between reference strains on the basis of phenotypic characters.

Character or test	*Beijerinckia* sp.	*Azomonas* sp.	*Klebsiella* sp.	*Azotobacter* sp.	*Azospirillum* sp.
Voges-Proskauer reaction	-	-	+	-	-
Colonies on potato dextrose agar	white	clear	white	yellow, orange, brown and white	pink, yellow and white
Utilization of various carbon sources					
- glucose	+	+	+	+	+
- mannitol	+	-	+	+	-
- rhamnose	-	-	+	+	-
- bensoate	-	-	-	-	-
- malate	-	-	-	-	+
- arabinose	+	-	+	-	-

Symbols : +, strains positive and -, strains negative.

To classify the free-living nitrogen fixing bacteria on the basis of biochemical assay the Voges-Poskaver reaction, utilizing of carbon sources such as glucose, mannitol, rhamnose, bensoate, arabinose and PDA were employed. The results from total 222 isolates along with biochemical assay could be classified as following; *Beijerinckia* sp. 16 isolates, *Klebsiella* sp. 4 isolates, *Azotobacter* sp. 1 isolate, *Azomonas* sp. 18 isolates, *Azospirillum* sp. 17 isolates and unidentified 166 isolates. Biochemical characteristic of free-living N_2-fixing bacteria each region was summarized in Table 5.

Table 5. Biochemical characteristic of free-living N_2-fixing bacteria

Genus	Total No. of isolate	No. of free-living nitrogen fixing bacterial strains			Location of isolation		
		North	Central	North-Eastern	North	Central	North-eastern
Beijerinckia sp.	16	4	1	11	IVNM331, IVNM341,IVNC24 and IVNC31	ICC*25	INEM123, INEM133, INEM244, IVNEM314, INECR37, IVNEM112, IVNEM211, IVNEM231, IVNEF31, VNED22 and VNEC*11
Klebsiella sp.	4	-	3	1	-	ICC42, IVCCR14 and IVCCR31	IVNEM133
Azotobacter sp.	1	-	-	1	-	-	INED23
Azomonas sp.	18	9	8	1	INM236, INM343, INCR11, INCR24, INR43, INF32 INC*16, IVNM311 and IVNF11	ICM222, ICM235, ICM321 ICF43, IVCM341 IVCR41, VCA12 and VCC*22	IVNEM341

Table 5. Continued

Genus	Total No. of isolate	No. of free-living nitrogen fixing bacterial strains			Location of isolation		
		North	Central	North-Eastern	North	Central	North-Eastern
Azospirillum sp.	17	5	11	1	INM111, INM314, INC29, INF16 and IVNB14	ICM137, ICM147, ICM249, ICM312, ICCR16, ICCR26, ICCR38, ICF15, ICF39, IVCM222 and VCD31	INEF14
Unidentified	166	57	51	58	-	-	-

Symbols: M1, Highest mountain; M2, Middle mountain; M3, Foot hill of mountain; CR, Rice in rotation with other crops; R, Rice cultivation; C, Field crop cultivation; F, Uncultivated; A, Undisturbed forest; B, Forest clearance for crop cultivation for 1-2 years; C* , Forest clearance for crop cultivation for 3 years; D, Intensive agricultural production using high rate of pesticides and fertilizers

3.2 *nif* D gene profile analysis

For studying *nif*D-PCR patterns of free-living nitrogen fixing bacteria from 222 isolates from 3 parts of Thailand showed *nif*D-PCR product patterns in Figure 3 to 25

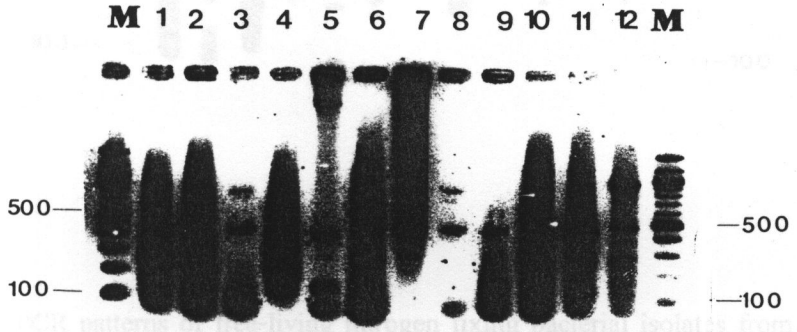

Figure 3. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from the highest mountain area in rainy season
Lane M, 100 bp. Ladder marker; Lane 1, INM11; Lane 2, INM125; Lane 3, INM133; Lane 4, INM145; Lane 5, ICM118; Lane 6, ICM123; Lane 7, ICM137; Lane 8, ICM147; Lane 9, INEM114; Lane 10, INEM123; Lane 11, INEM133 and Lane 12, INEM143

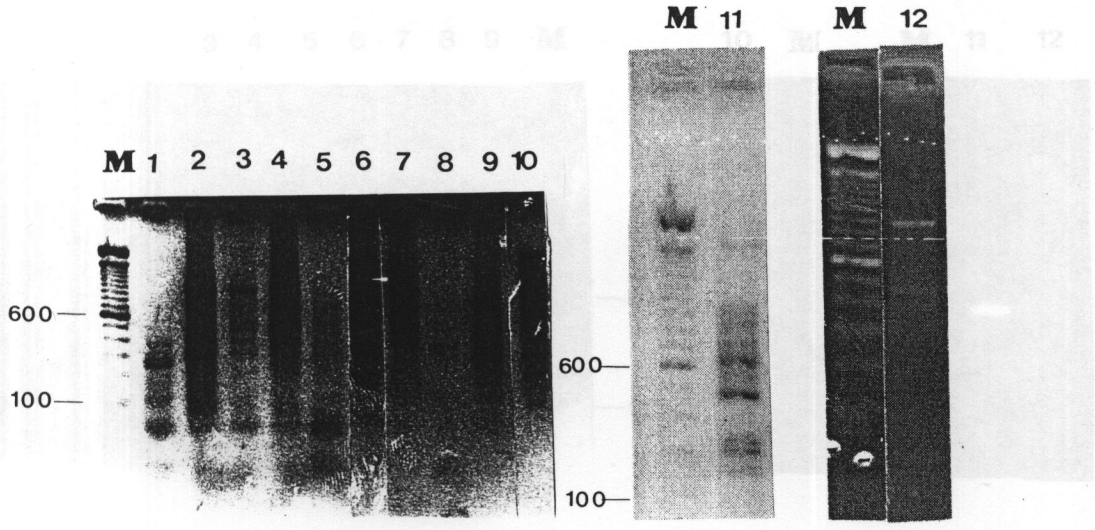

Figure 4. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from the highest mountain area in dry season
Lane M, 100 bp. Ladder marker; Lane 1, IVNM116; Lane 2, IVNM125; Lane 3, IVNM135; Lane 4, IVNM142; Lane 5, IVCM113; Lane 6, IVCM123; Lane 7, IVCM131; Lane 8, IVNEM112; Lane 9, IVNEM121; Lane 10, IVNEM133; Lane 11, IVCM144 and Lane 12, IVNEM141

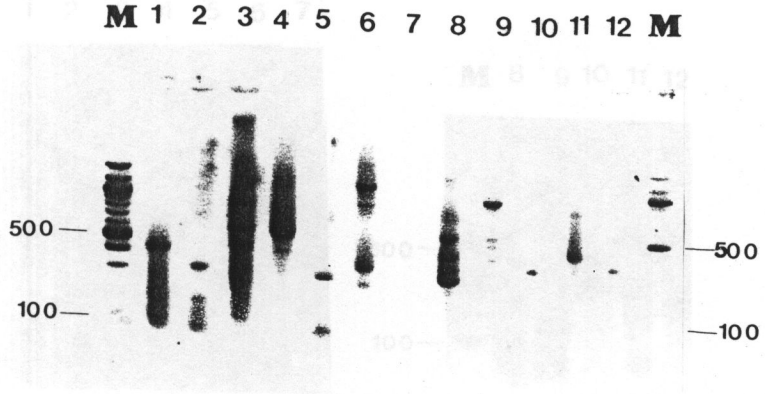

Figure 5. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from the middle mountain area in rainy season
Lane M, 100 bp. Ladder marker; Lane 1, INM213; Lane 2, INM224; Lane 3, INM236; Lane 4, INM248; Lane 5, ICM219; Lane 6, ICM222; Lane 7, ICM235; Lane 8, ICM249; Lane 9, INEM216; Lane 10, INEM223; Lane 11, INEM236 and Lane 12, INEM244

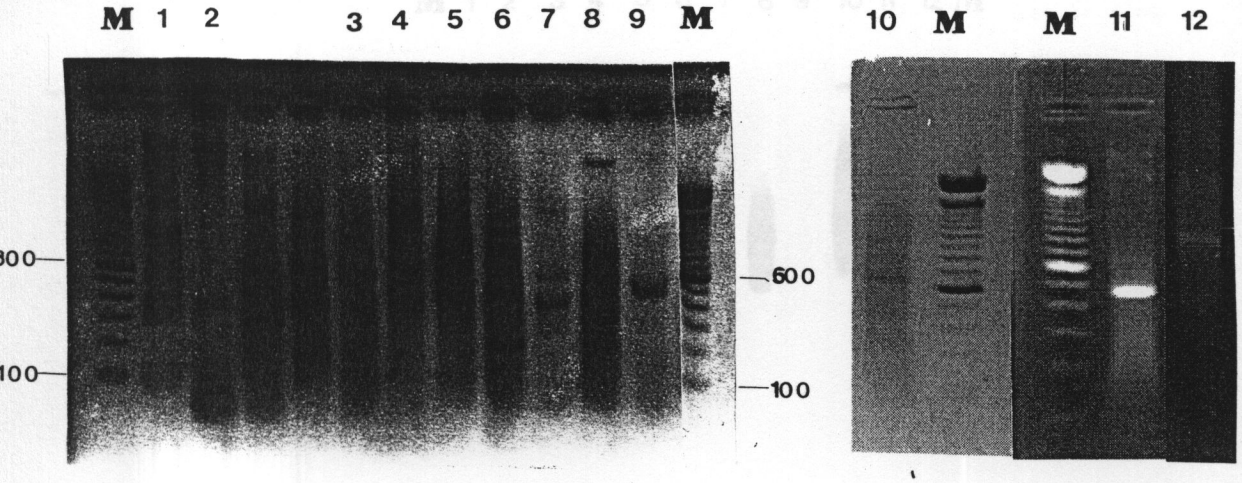

Figure 6. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from the middle mountain area in dry season
Lane M, 100 bp. Ladder marker; Lane 1, IVNM212; Lane 2, IVNM224; Lane 3, IVCM213; Lane 4, IVNM222; Lane 5, IVCM233; Lane 6, IVCM243; Lane 7, IVNEM211; Lane 8, IVNEM224; Lane 9, IVNEM233; Lane 10, IVNEM241; Lane 11, IVNM243 and Lane 12, IVNM231

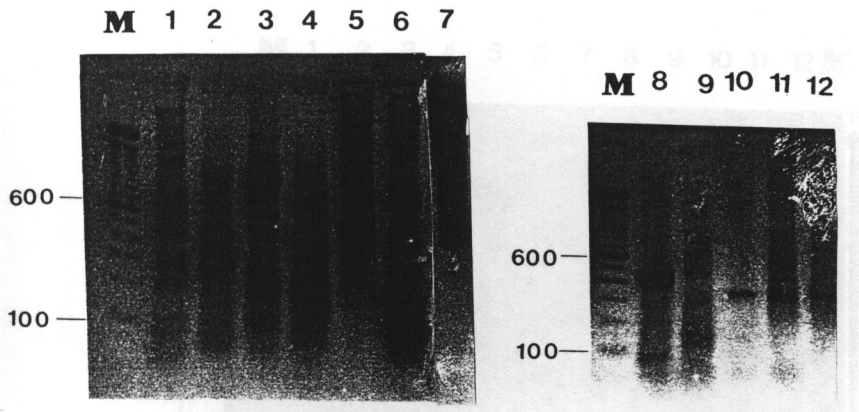

Figure 7. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from foot hill of mountain area in rainy season
Lane M, 100 bp. Ladder marker; Lane 1, INM314; Lane 2, INM324; Lane 3, INM336; Lane 4, INM343; Lane 5, ICM312; Lane 6, ICM321; Lane 7, ICM332; Lane 8, ICM347; Lane 9, INEM314; Lane 10, INEM323; Lane 11, INEM332 and Lane 12, INEM345

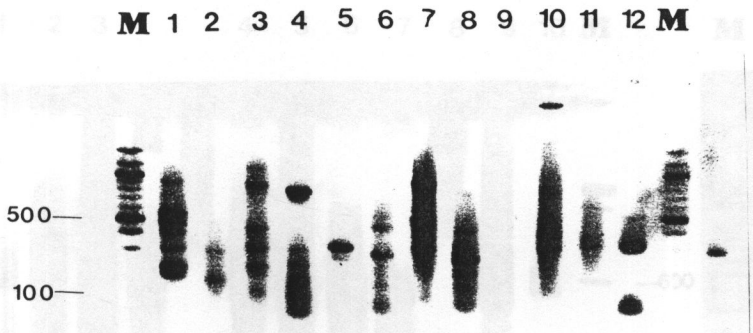

Figure 8. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from foot hill of mountain area in dry season
Lane M, 100 bp. Ladder marker; Lane 1, IVNM311; Lane 2, IVNM322; Lane 3, IVNM331; Lane 4, IVNM341; Lane 5, IVCM315; Lane 6, IVCM323; Lane 7, IVCM335; Lane 8, IVCM341; Lane 9, IVNEM314; Lane 10, IVNEM324; Lane 11, IVNEM333 and Lane 12, IVNEM341

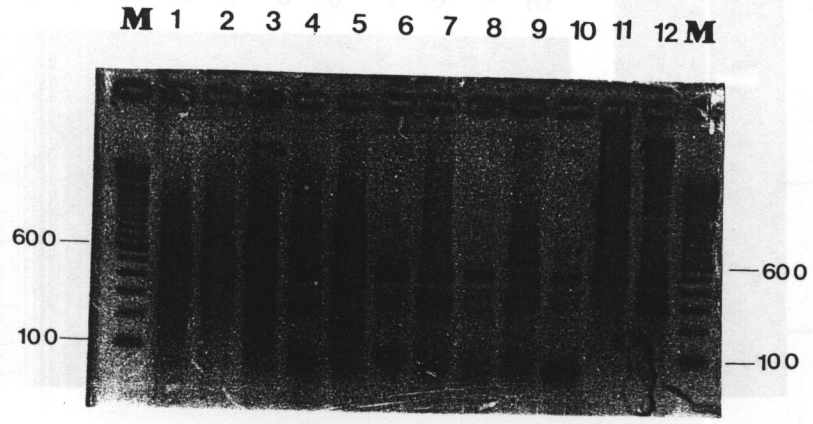

Figure 9. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from rice in rotation with other crops area in rainy season
Lane M, 100 bp. Ladder marker; Lane 1, INCR11; Lane 2, INCR24; Lane 3, INCR36; Lane 4, INCR410; Lane 5, ICCR16; Lane 6, ICCR26; Lane 7, ICCR38; Lane 8, ICCR41; Lane 9, INECR11; Lane 10, INECR22; Lane 11, INECR37 and Lane 12, INECR44

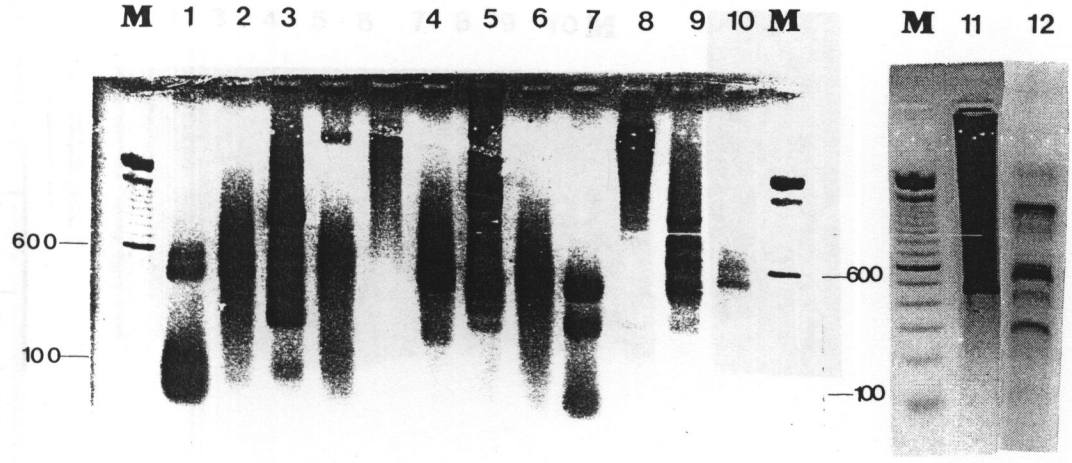

Figure 10. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from rice in rotation with other crops area in dry season
Lane M, 100 bp. Ladder marker; Lane 1, IVNCR15; Lane 2, IVNCR22; Lane 3, IVNCR34; Lane 4, IVCCR21; Lane 5, IVCCR31; Lane 6, IVCCR41; Lane 7, IVNECR12; Lane 8, IVNECR22; Lane 9, IVNECR33; Lane 10, IVNECR44; Lane 11, IVNCR43 and Lane 12, IVCCR14

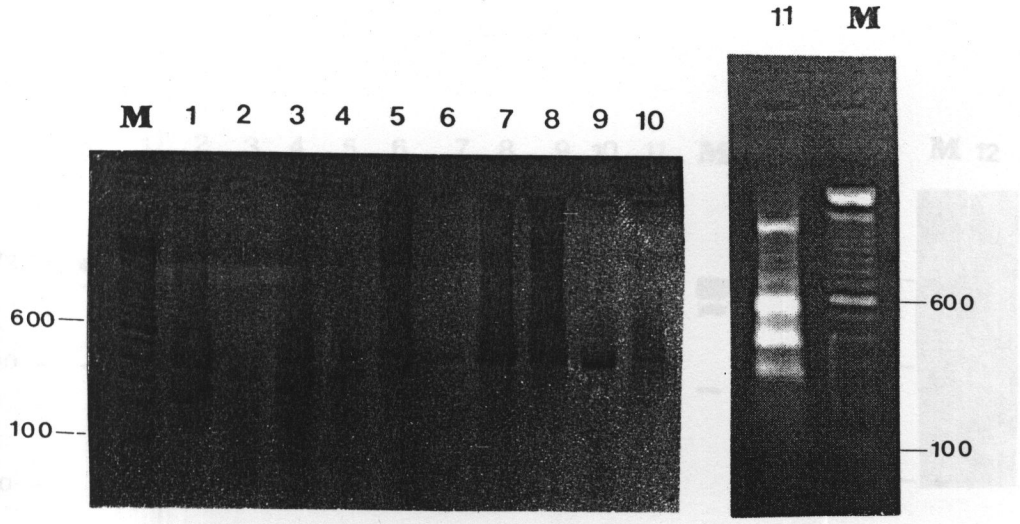

Figure 11. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from rice cultivation area in rainy season
Lane M, 100 bp. Ladder marker; Lane 1, INR12; Lane 2, INR25; Lane 3, INR33; Lane 4, INR43; Lane 5, ICR12; Lane 6, ICR24; Lane 7, ICR35; Lane 8, ICR44; Lane 9, INER15; Lane 10, INER24 and Lane 11, INER46

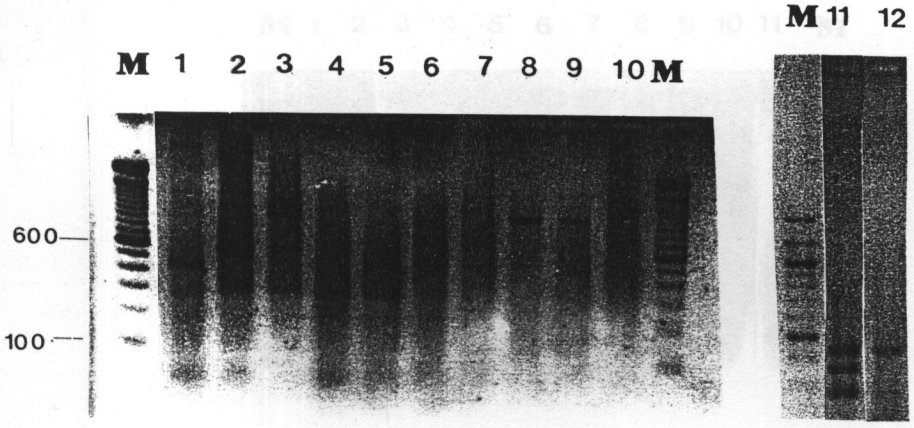

Figure 12. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from rice cultivation area in dry season
Lane M, 100 bp. Ladder marker; Lane 1, IVNR32; Lane 2, IVNR44; Lane 3, IVCR12; Lane 4, IVCR21; Lane 5, IVCR33; Lane 6, IVCR41; Lane 7, IVNER11; Lane 8, IVNER21; Lane 9, IVNER31and Lane 10, IVNER42; Lane 11, IVNR13 and Lane 12, IVNR26

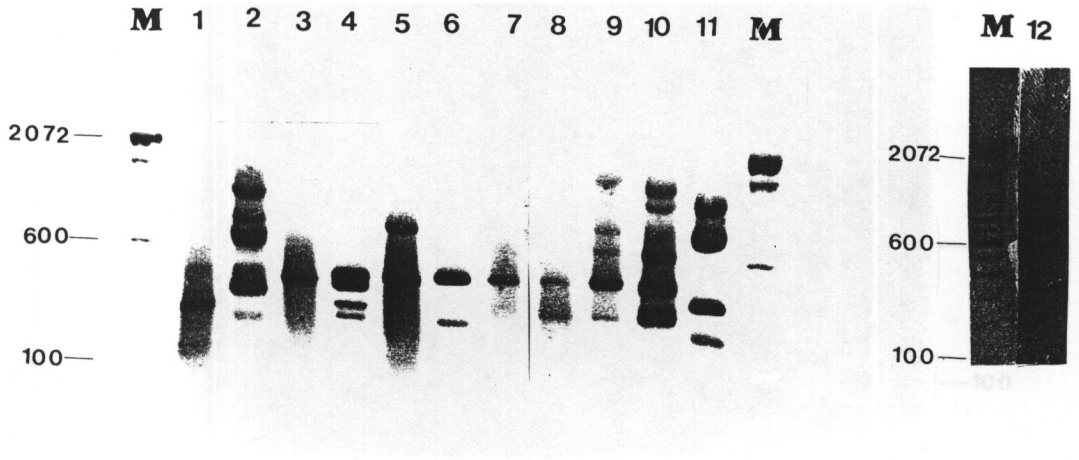

Figure 13. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from crop cultivation area in rainy season
Lane M, 100 bp. Ladder marker; Lane 1, INC15; Lane 2, INC29; Lane 3, INC31; Lane 4, INC45; Lane 5, ICC11; Lane 6, ICC25; Lane 7, ICC34; Lane 8, INEC14; Lane 9, INEC23; Lane 10, INEC48; Lane 11, INEC33 and Lane 12, ICC42

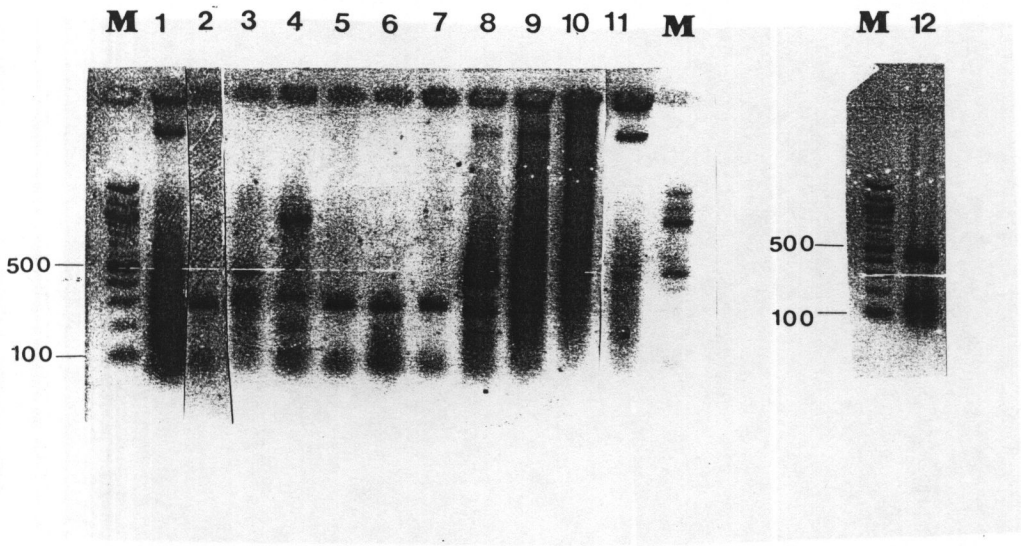

Figure 14. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from crop cultivation area in dry season
Lane M, 100 bp. Ladder marker; Lane 1, IVNC13; Lane 2, IVNC24; Lane 3, IVNC31; Lane 4, IVNC41; Lane 5, IVCC13; Lane 6, IVCC24; Lane 7, IVCC32; Lane 8, IVCC45; Lane 9, IVNEC12; Lane 10, IVNEC22; Lane 11, IVNEC42 and Lane 12, IVNEC33

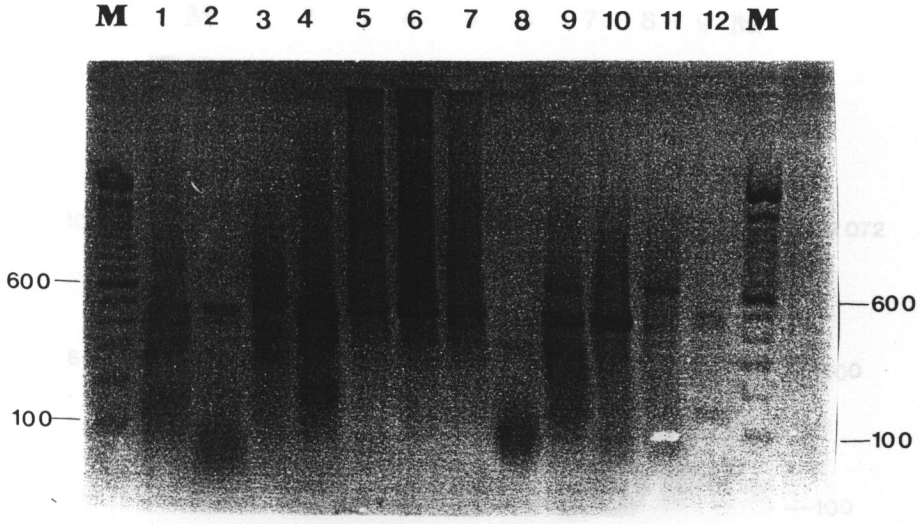

Figure 15. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from uncultivated area in rainy season
Lane M, 100 bp. Ladder marker; Lane 1, INF16; Lane 2, INF25; Lane 3, INF32; Lane 4, INF47; Lane 5, ICF15; Lane 6, ICF24; Lane 7, ICF39; Lane 8, ICF43; Lane 9, INEF14; Lane 10, INEF27; Lane 11, INEF33 and Lane 12, INEF47

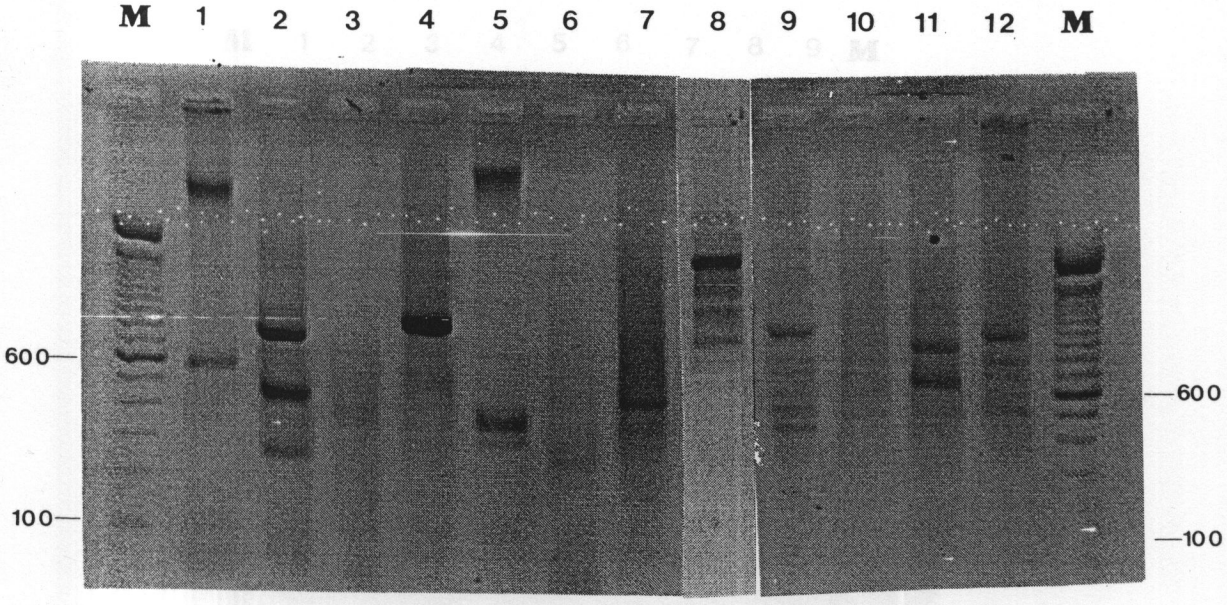

Figure 16. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from uncultivated area in dry season
Lane M, 100 bp. Ladder marker; Lane 1, IVNF11; Lane 2, IVNF24; Lane 3, IVNF31; Lane 4, IVNF41; Lane 5, IVCF13; Lane 6, IVCF24; Lane 7, IVCF32; Lane 8, IVCF42; Lane 9, IVNEF12; Lane 10, IVNEF24; Lane 11, IVNEF31 and Lane 12, IVNEF42

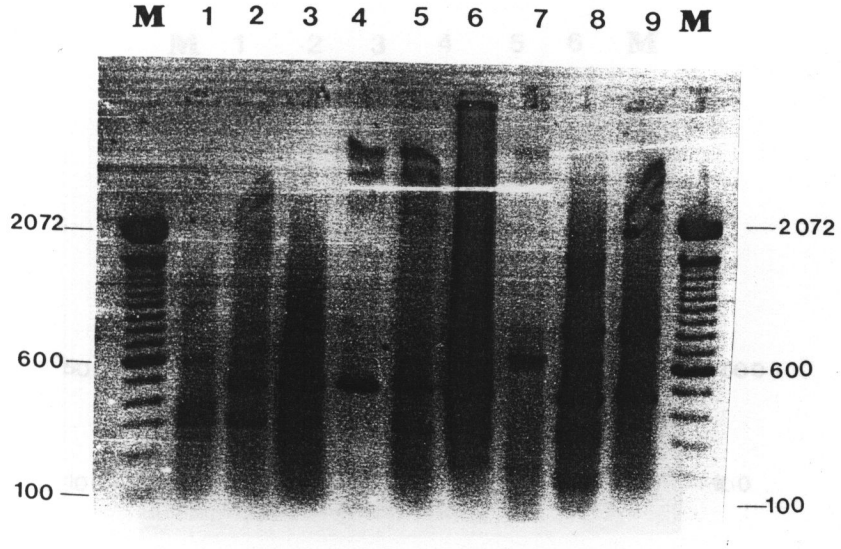

Figure 17. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from intensive agriculture production using high rate of pesticides and fertilizer area in rainy season Lane M, 100 bp. Ladder marker; Lane 1, IND13; Lane 2, IND24; Lane 3, IND39; Lane 4, ICD13; Lane 5, ICD21; Lane 6, ICD31; Lane 7, INED17; Lane 8, INED23 and Lane 9, INED33

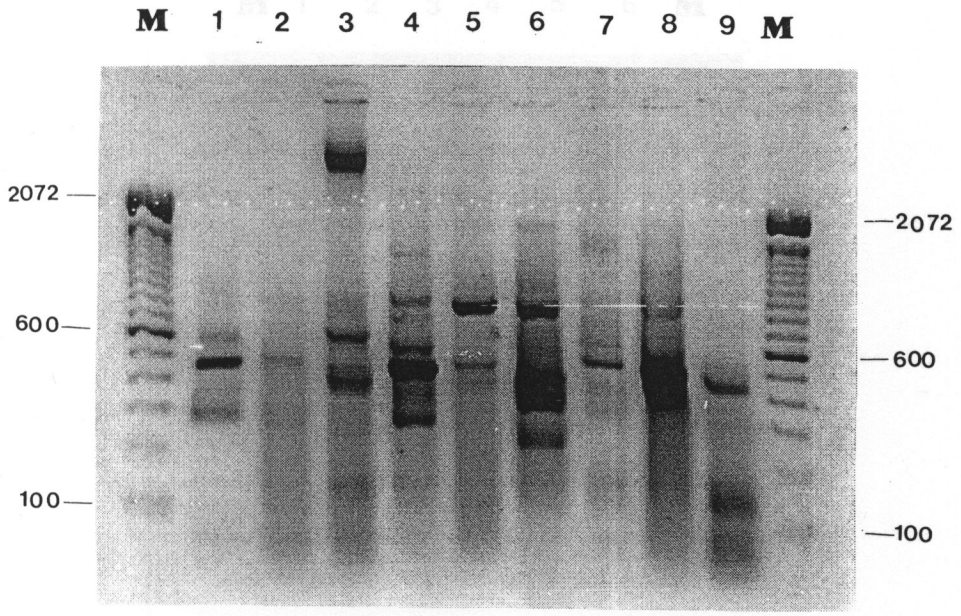

Figure 18. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from intensive agriculture production using high rate of pesticides and fertilizer area in dry season Lane M, 100 bp. Ladder marker; Lane 1, VND13; Lane 2, VND26; Lane 3, VND31; Lane 4, VCD11; Lane 5, VCD21; Lane 6, VCD31; Lane 7, VNED11; Lane 8, VNED22 and Lane 9, VNED37

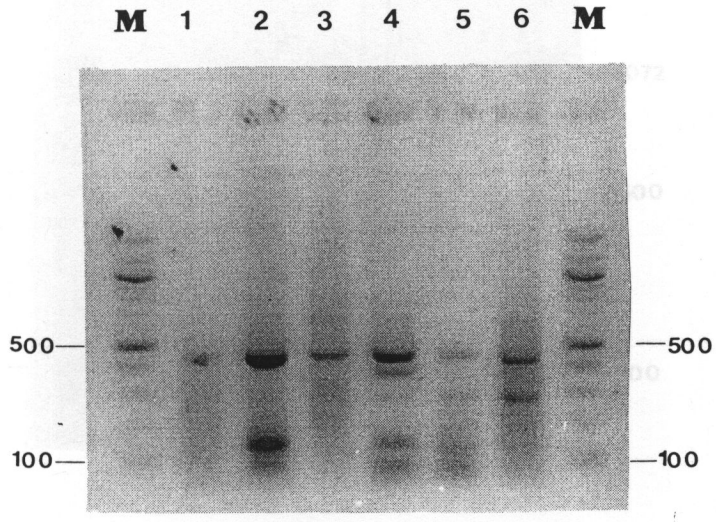

Figure 19. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from undisturbed forest area in rainy season
Lane M, 100 bp. Ladder marker; Lane 1, INA19; Lane 2, INA23; Lane 3, ICA16; Lane 4, ICA23; Lane 5, INEA17 and Lane 6, INEA27

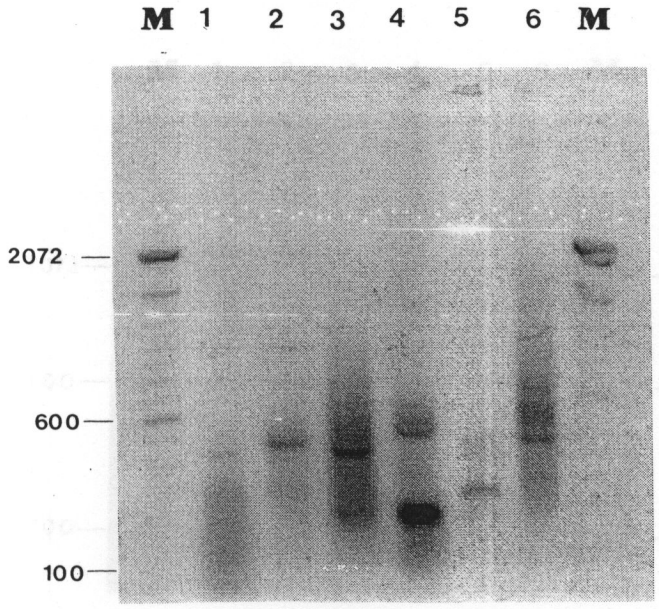

Figure 20. *nif*D-PCR[2] patterns of free-living nitrogen fixing bacterial isolates from undisturbed forest area in dry season
Lane M, 100 bp. Ladder marker; Lane 1, VNA13; Lane 2, VNA23; Lane 3, VCA12; Lane 4, VCA22; Lane 5, VNEA11 and Lane 6, VNEA21

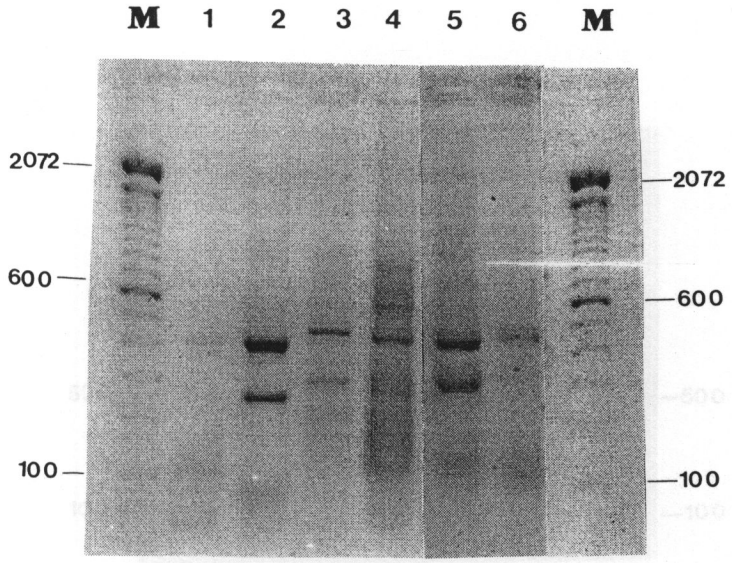

Figure 21. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from forest clearance from crop cultivation from 1-2 years area in rainy season
Lane M, 100 bp. Ladder marker; Lane 1, INB13; Lane 2, INB24; Lane 3, ICB12; Lane 4, ICB27; Lane 5, INEB11 and Lane 6, INEB21

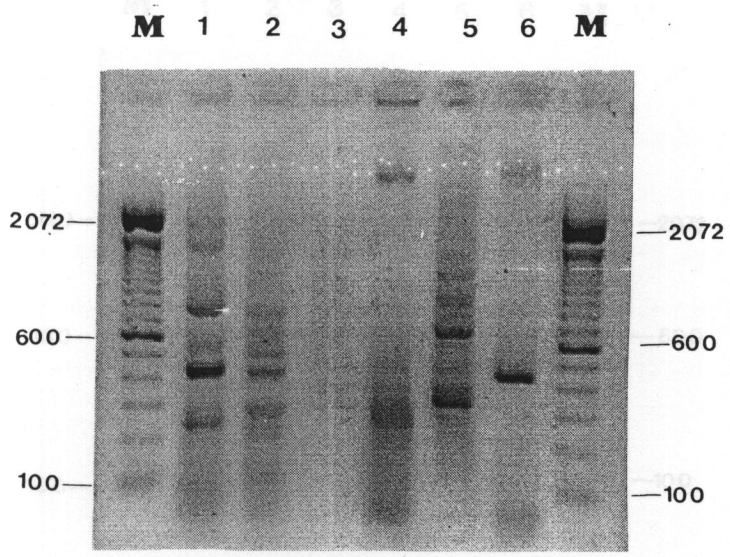

Figure 22. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from forest clearance from crop cultivation from 1-2 years area in dry season
Lane M, 100 bp. Ladder marker; Lane 1, VNB14; Lane 2, VNB21; Lane 3, VCB11; Lane 4, VCB23; Lane 5, VNEB14 and Lane 6, VNEB21

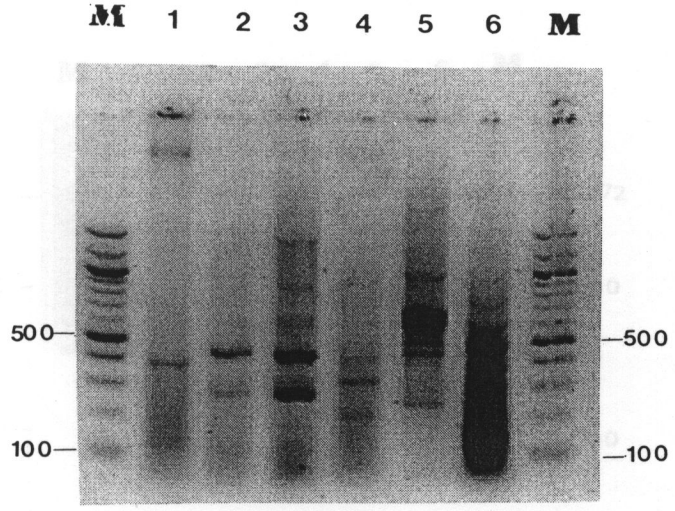

Figure 23. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from crop cultivation from 3 years area in rainy season
Lane M, 100 bp. Ladder marker; Lane 1, INC*16; Lane 2, INC*28; Lane 3, ICC*13; Lane 4, ICC*25; Lane 5, INEC*16 and Lane 6, INEC*23

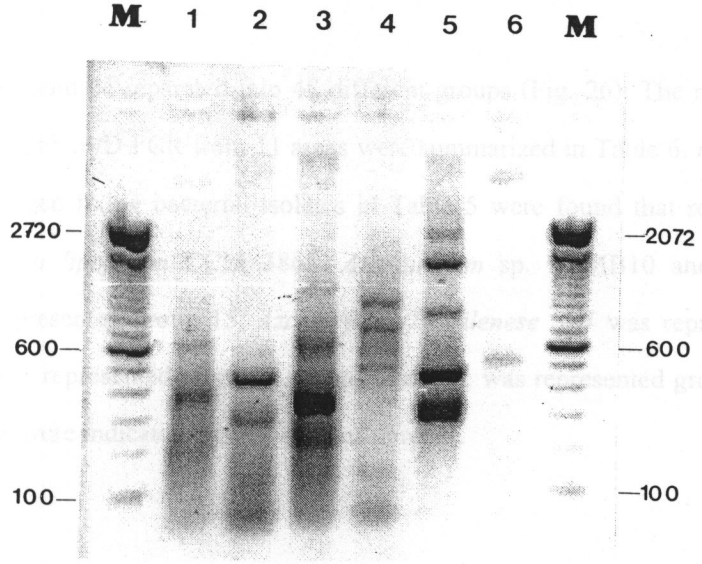

Figure 24. *nif*D-PCR patterns of free-living nitrogen fixing bacterial isolates from crop cultivation from 3 years area in dry season
Lane M, 100 bp. Ladder marker; Lane 1, VNC*12; Lane 2, VNC*24; Lane 3, VCC*11; Lane 4, VCC*22; Lane 5, VNEC*11 and Lane 6, VNEC*21

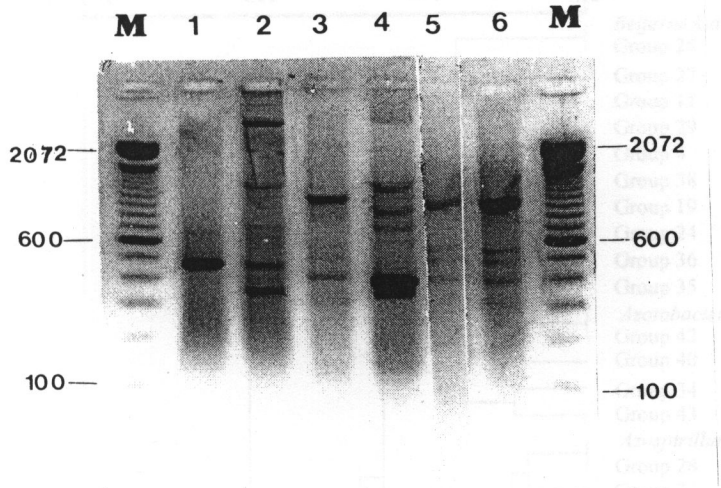

Figure 25. *nif*D-PCR patterns of free-living nitrogen fixing bacteria
Lane M, 100 bp. Ladder marker; Lane 1, *Beijerinckia* sp.; Lane 2, *Azotobacter* sp.; Lane 3, *Azospirillum lipoferum* CCM3863; Lane 4, *Azospirillum brasilense* Sp7; Lane 5, *Azospirillum* sp. UPMB10 and Lane 6, *Azospirillum* sp. UPMB13

The results could be separated into 48 different groups (Fig. 26). The results of the group categorized along with *nif*D-PCR from 11 areas were summarized in Table 6. *nif*D–PCR products of free-living nitrogen fixing bacteriai isolates in Table 5 were found that reference strains; as strains; *Azospirillum lipoferum* CCM 3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 were represented group 15, *Azospirillum brasilenese* Sp7 was represented group 28, *Beijerinckia* sp. was represented group 25, *Azotobacter* sp. was represented group 42. Group 6 of *nif*D-PCR patterns were indicated highest isolates number.

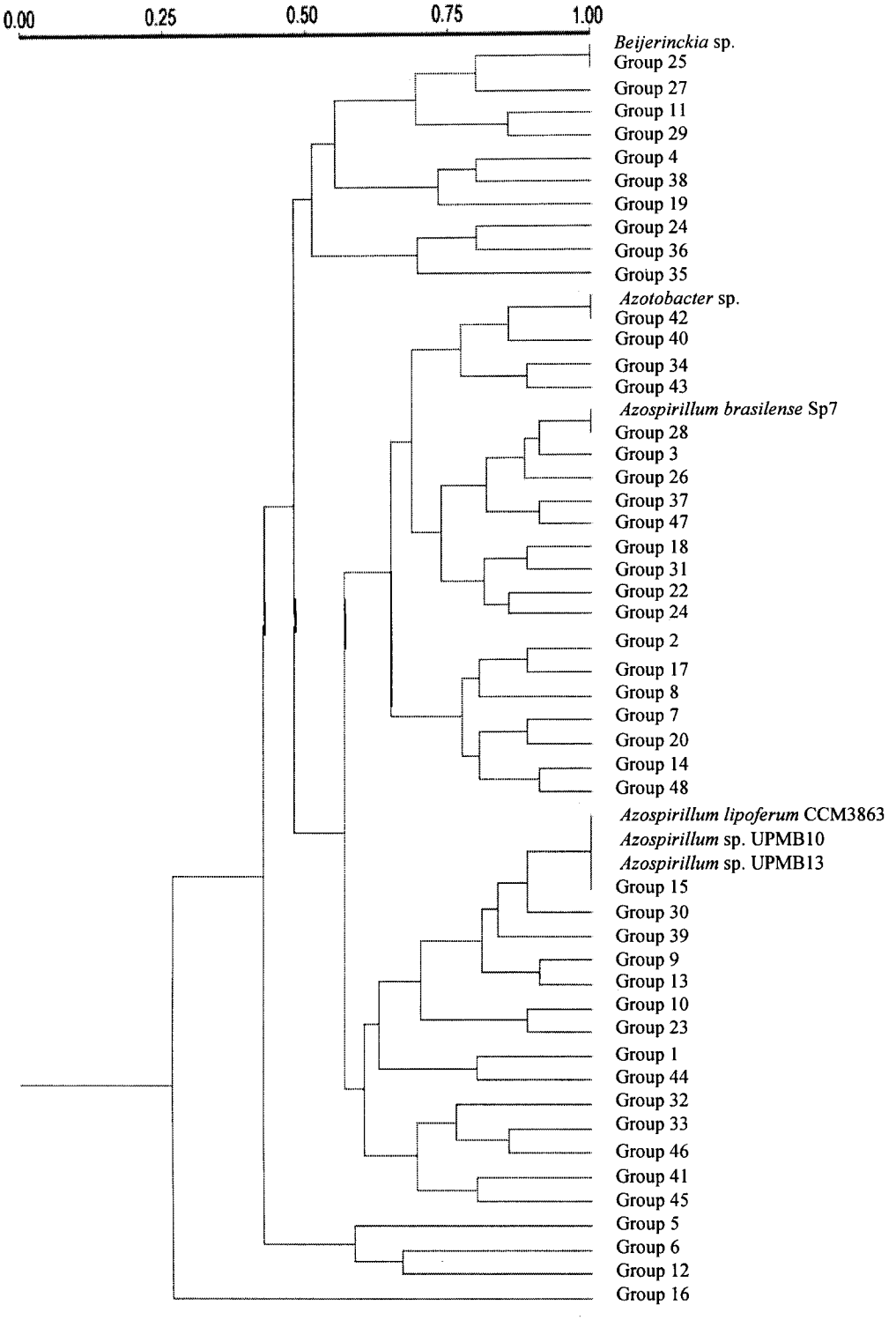

Figure 26. Dendrogram of 48 groups of free-living nitrogen fixing bacteria

Table 6. *nif* D- PCR pattern of free-living nitrogen fixing bacteria was separated into different 48 groups from 3 parts of Thailand

Group	No. of isolate	Location		
		North	Central	North-Eastern
1	43	INM111 (AS), INM125 INCR11(AM), INCR24 (AM) INR33, INR43 (AM) IND13, IND24 INF47 INB24 INC*28 IVNC13, INC31 IVNEM116 IVNR32, IVNR44 VNC*24	ICM235 (AM) ICA16 ICCR26 (AS), ICCR38 (AS) ICB12, ICB27 ICD21, ICD31 IVCF24 IVCCR21 IVCR21, IVCR33	INEM236 INEA17, INEA27 INEC14 INEF47 INECR22, INECR44 INEB11 IVNEM133 IVNEM314 (B), IVNEM333, IVNEM341 (AM), IVNEM133 (K) IVNEC33 IVNECR12
2	2	INM145	ICM249 (AS)	-
3	3	-	ICM123	INED23 (K) IVNECR33
4	11	INM133 IVNF41	ICM147 (AS), ICM321 (AM) ICC11 VCD21 VCB11	INED33 IVNEM224, IVNEM112 (B) VNED22 (B)
5	4	-	ICM118 VCR12 (AM)	VNEA11 VNED37
6	46	INCR36 INC31 INF25 IND39 INA19 INB13 INC*16 (AM) IVNC24 (B) IVNF31 IVNCR15, IVNCR43	ICM137 (AS), ICM332 ICM312 (AS), ICM347 ICCR48 ICR12, ICR24, ICR35 ICC34 ICF15 (AS), ICF24, ICF39 (AS)	INEM114, INEM323 INECR37 (B) INER15, INER24 INEF27 INED17 INEB21 IVNECR45 IVNER41 IVNEC12, IVNEC22, IVNEC42 IVNEF24

Table 6. Continued

Group	No. of isolate	Location		
		North	Central	North-Eastern
		IVNR26 VNA13	ICD13 IVCF32 IVCCR41 IVCR41 (AM)	VNED11 VNEB21 VNEC*21
7	2	INM248 IVNM331 (B)	-	-
8	6	INM213 INA23 INF47	VCA22	INEM133 (B) INEM314
9	1	IVNM212	-	-
10	1	-	IVCM243	-
11	2	-		IVNEM241 IVNER31
12	1	-	IVCF13	-
13	1	-	IVCCR31 (K)	
14	1	-	-	INEM123 (B)
15	1	-	-	INEM143 *Reference strains; *Azospirillum lipoferum* CCM 3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13
16	11	IVNM224 INR25 IVNM224	ICM219, ICM312 ICF43 (AM) VCC13, IVCC24, IVCC32 VCB23	INEM223 IVNEM231 (B) IVNECR22
17	2	INM236 (AM) INC15	-	-
18	1	-	VCD31 (AS)	-
19	1	IVNCR22	-	-
20	1	IVNCR34-	-	-
21	1	-	IVCM315	-

Table 6. Continued

Group	No. of isolate	Location		
		North	Central	North-Eastern
22	9	IVNF24 VND26	ICCR16 (AS) IVCM323, IVCM341 (AM), IVCM233	INEM244 (B), INEM345 IVNEM324
23	4	IVNM311 (AM), IVNM322, IVNM341 (B) IVNC41	-	-
24	2	INM314	IVCC45	
25	6	IVNM243 IVNF11 (AM) VNC*12	ICR44	INEF33 IVNEM211 (B) *Reference strain; *Beijerinckia* sp.
26	1	INC29 (AS)	-	-
27	1	-	IVCM213	-
28	5	-	VCD11	INEC27, INEC33 IVNEF12 VNEB14 *Reference strain; *Azospirillum brasilenese* Sp 7
29	1	-	IVCF42	-
30	1	-	IVCM335	-
31	4	INCR410 VNB14 (AS)	ICC*13	INER46
32	1	-	-	IVNEF42
33	4	VNB21	ICM222 (AM)	INEC47 INEC*16
34	1	-	-	INEM332
35	2	-	ICC*25 (B) VCC*11	-
36	1	-	-	VNEC*11 (B)
37	1	INM324	-	-
38	7	INM336 VND31	ICC42 (K) VCC*22 (AM)	INEM216 INEC*23 IVNEF31 (B)

Table 6. Continued

Group	No. of isolate	Location		
		North	Central	North-Eastern
39	5	INC45 INF16 (AS) IVNF14	ICA23 ICC25	-
40	2		IVCM144	IVNER11
41	3	VNA23	-	IVNEM141 IVNER21
42	7	INM343 (AM) INF32 (AM) VND13	-	INECR11 INEF14 (AS) IVNEM121 VNEA21 *Reference strain; *Azotobacter* sp.
43	1	-	-	IVNEM121
44	3	INR12 IVNM232	IVCM222 (AS)	-
45	4	IVNM125, IVNM134, IVNM142	IVCM113	-
46	1	-	IVCM131	-
47	1	-	IVCCR14 (K)	-
48	1	-	IVCM123	-

Notes : Results of biochemical assay : AS, *Azospirillum* sp.; AM, *Azomonas* sp. ; B, *Beijerinckia* sp. ; K, *Klebsiella* sp.
Result of *nif*D–PCRproducts: *reference strain; *Azospirillum lipoferum* CCM 3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 were represented group 15, *A. brasilenese* Sp 7 represented group 28, *Beijerinckia* sp. was represented group 25, *Azotobacter* sp. was represented group 42.

Symbols: M1, Highest mountain; M2, Middle mountain; M3, Foot hill of mountain; CR, Rice in rotation with other crops; R, Rice cultivation; C, Field crop cultivation; F, Uncultivated; A, Undistrub forest; B, Forest clearance for crop cultivation for 1-2 years; C*, Forest clearance for crop cultivation for 3 years; D, Intensive agricultural production using high rate of pesticides and fertilizers

3.2.1 nif D-PCR of free-living nitrogen fixing bacteria from the highest mountain area

The PCR product was mainly found the major band in size of 450 bp.. Comparison of free-living nitrogen fixing bacteria by *nif* D patterns from 3 regions; the Central, the North and the North Eastern in 2 seasons were analyzed. Dendrogram bacterial isolates between rainy and dry season in the North was shown that the similar strains in rainy season and dry season as INM111, INM125 and IVNM116 were found closely related to *Beijerinckia* sp. and *Azotobacter* sp. The bacterial strain in rainy season as INM145 was found closely related to *Azotobacter* sp.. The similar strains in dry season as IVNM125, IVNM134 and IVNM145 were shown closely related to *Azospirillum brasilense* Sp7, *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. For isolate in rainy season as INM133 was in another different group. Bacterial strain in rainy season and dry season as INM111 and IVNM116 were found as the dominant native strains (Fig. 27). Dendrogram in the Central indicated that strains as IVCM144 in dry season and ICM118 in rainy season were found closely related to *Beijerinckia* sp., *Azotobacter* sp. and *Azospirillum brasilense* Sp7. The bacterial strains in dry season as IVCM131, IVCM123 and IVCM113 were displayed closely related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. ICM118, ICM137 and ICM147 strains in rainy season were in another different group (Fig. 28). Dendrogram in the North Eastern in Fig. 4, found that the similar strains in rainy season and dry season as INEM133 and IVNEM133 were found closely related to *Beijerinckia* sp. and *Azotobacte* sp. and strains as INEM133 and IVNEM133 were assumed to be dominant native strains. Bacterial stains in rainy season and dry season as INEM143, INEM123, IVNEM121, IVNEM141 and IVNEM112 were indicated closely related to *Azospirillum brasilense* Sp7, *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. For strain in rainy season as INEM114 was in another different group (Fig. 29).

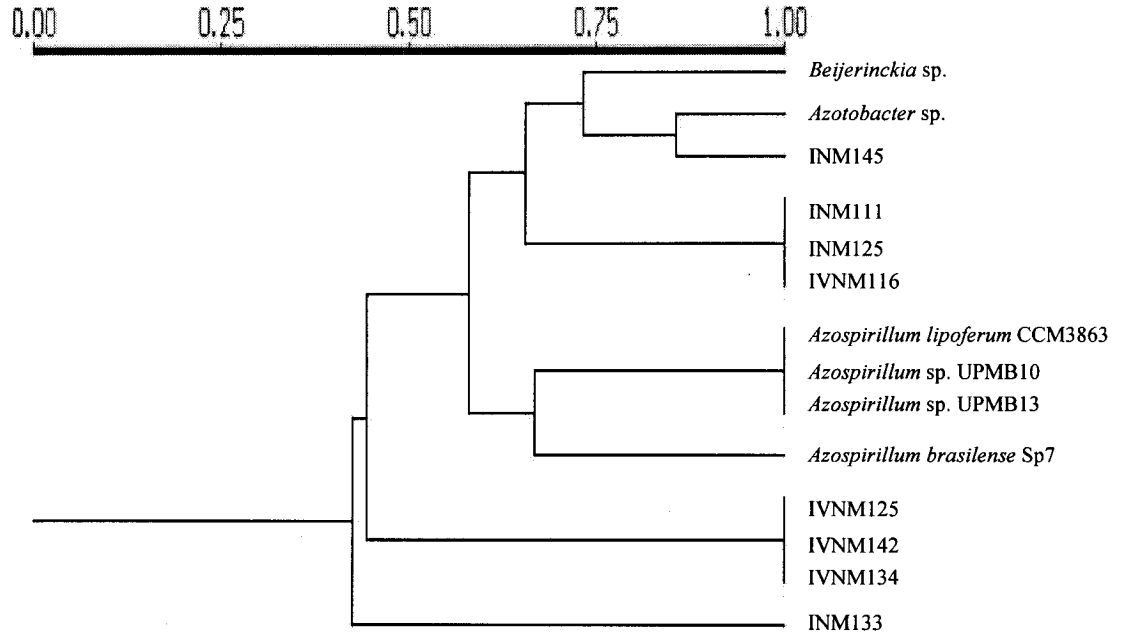

Figure 27. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North region from the highest mountain area.

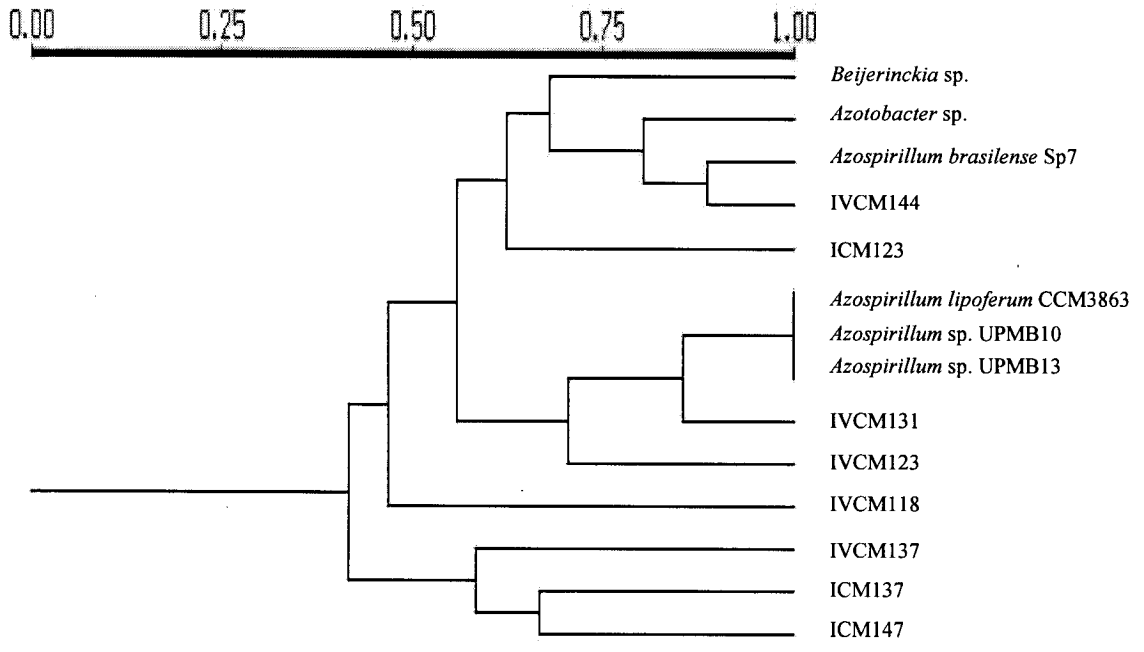

Figure 28. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the Central region from the highest mountain area.

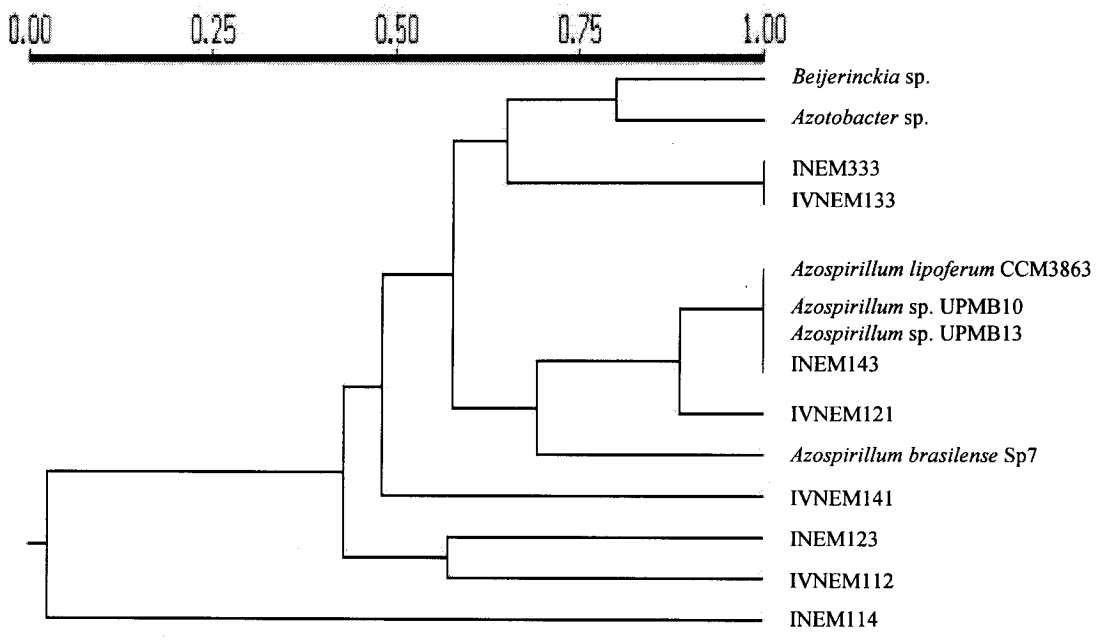

Figure 29. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North Eastern region from the highest mountain area

3.2.2 nifD-PCR of free-living nitrogen fixing bacteria from the middle mountain area

The PCR patterns of isolated samples from the middle mountain in rainy and dry season were found different stains among 3 regions. Dendrogram of isolate in the North showed that bactrial strain in dry season was similar to *Beijerinckia* sp. Isolated strain in both seasons as INM236, INM213 and IVNM232 were found nearly related to *Azotobacter* sp. IVNM212 strain in dry season was indicated closely related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. INM248 strain in rainy season was found closely related to *A.brasilenese* Sp7. The similar strain and found the dominant native strains as INM224 in rainy season (Fig. 30). Dendrogram of the Central region found that the strain in dry season as IVCM213 was same to *Beijerinckia* sp. and the strain in rainy season as ICM249 was indicated similar to *Azotobacter* sp.. The bacterial strain in dry season as IVCM243 was displayed closely related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. Isolated strains in both season as ICM222, ICM235, IVCM233 and IVCM222 were found closely related to *A.brasilenese* Sp7. The bacterial isolate in rainy season as ICM219 was

in another different group (Fig. 31). Dendrogram of the North Eastern of strain IVNEM211 in dry season was similar to *Beijerinckia* sp.. The similar strains in both season as INEM216 and IVNEM241 were found closely related to *Beijerinckia* sp. and *Azotobacter* sp.. Strains in rainy season as INEM236 and INEM244 were indicated closely related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. The similar strains in both season as INEM223 and IVNEM231 and strain IVNEM224 in dry season were in another different group (Fig. 32).

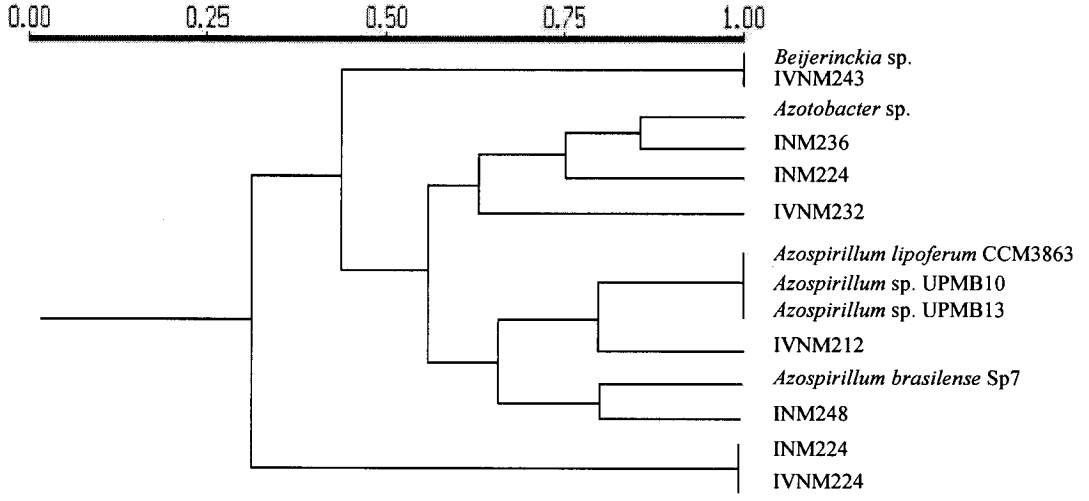

Figure 30. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North region from the middle mountain area

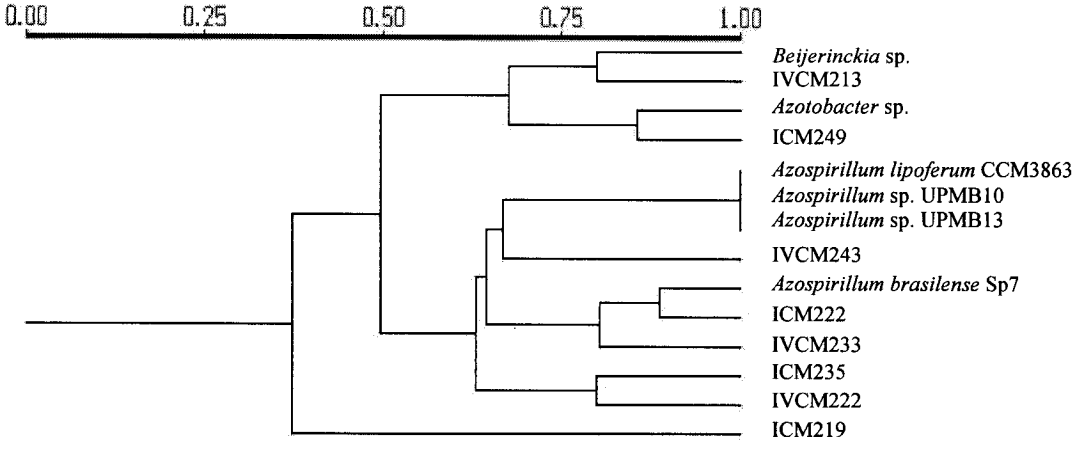

Figure 31. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the Central region from the middle mountain area

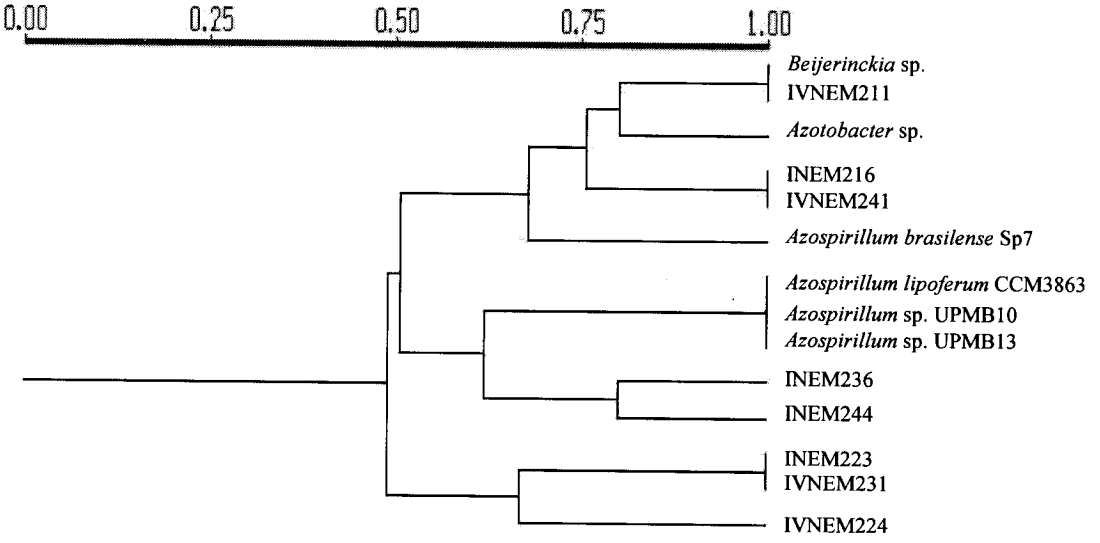

Figure 32. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North Eastern region from the middle mountain area

3.2.3 nif D PCR of free-living nitrogen fixing bacteria from foot hill of mountain area

The Dendrogram in the North region showed that INM343 strain in rainy season was close to *Azotobacter* sp.. Bacterial isolated strains in rainy season as INM314, INM324 and INM336 were found closely related to *Azospirillum brasilenese* Sp7. IVNM311 strain in dry season was displayed closely related to *A.lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. Similar strain as IVNM322 and IVNM341 and IVNM331 were observed only in dry season (Fig. 33). Dendrogram in the Central indicated the similar bacterial strains in rainy season such as ICM312, ICM332 and ICM347 were found closely related to *Beijerinckia* sp. and *Azotobacter* sp.. IVCM335 strain in dry season was shown closely related to *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. The similar isolated samples in dry season as IVCM323 and IVCM341, IVCM315 strain in dry season and ICM321 in rainy season were found closed related to *A.brasilenese* Sp7 (Fig. 34). Dendrogram in the North Eastern found that INCM332 strain was same to *Azotobacteria* sp. and indicated closely related to *Beijerinckia* sp.. Group similar strains in dry season as IVNEM314, IVNEM341 and IVNEM333 were found closely related to *Beijerinckia* sp., *Azotobacteria* sp. and *A. brasilenese* Sp7. Similar isolates in rainy season and dry season as INEM345 and IVNEM324 and INEM314

strain in rainy season were found closely related to *Beijerinckia* sp., *Azotobacteria* sp. and *A. brasilenese* Sp7. Strain as INEM323 was belong in an another different groups (Fig. 35).

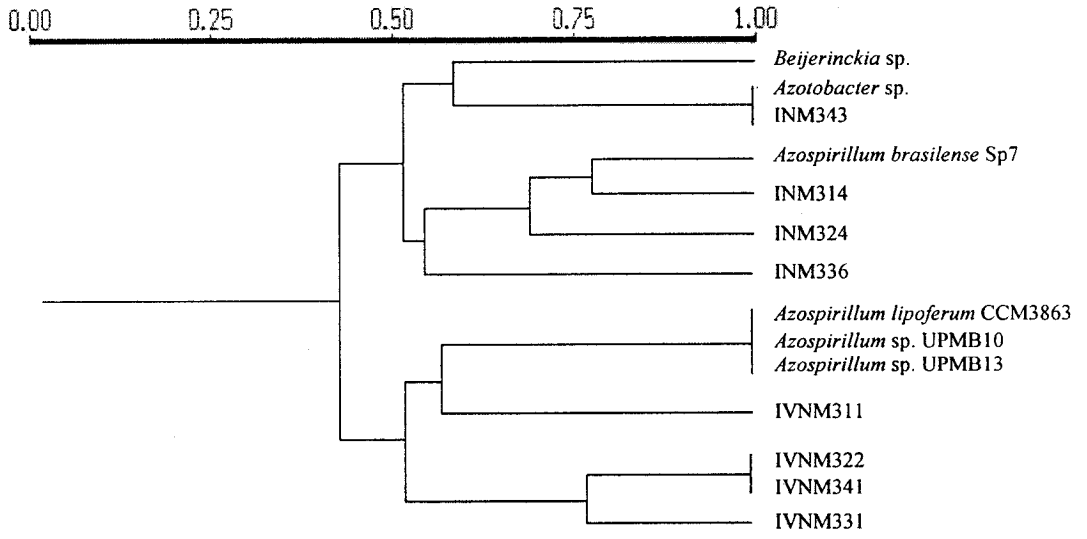

Figure 33. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North region from foot hill of mountain area

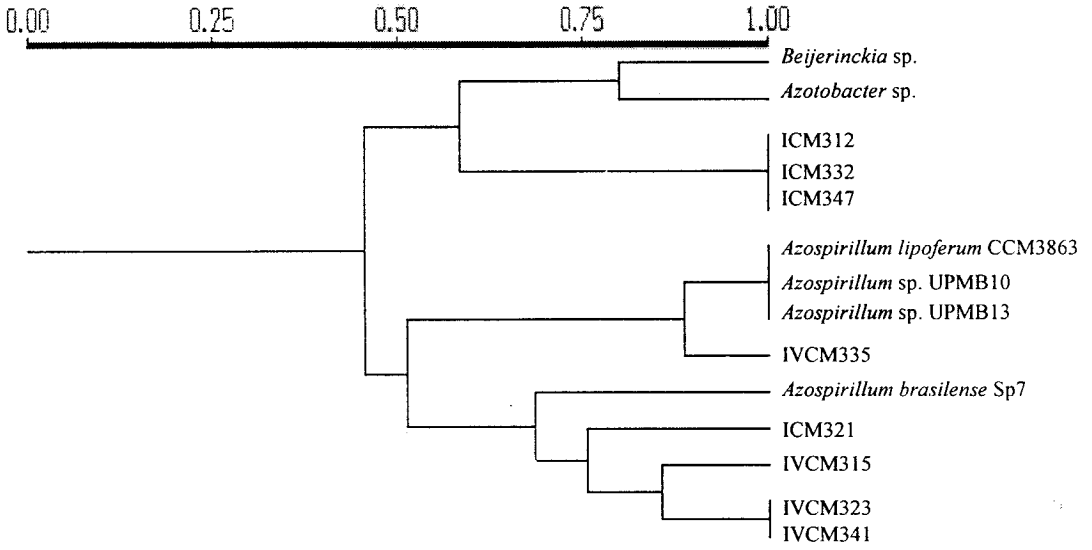

Figure 34. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the Central region from foot hill of mountain area

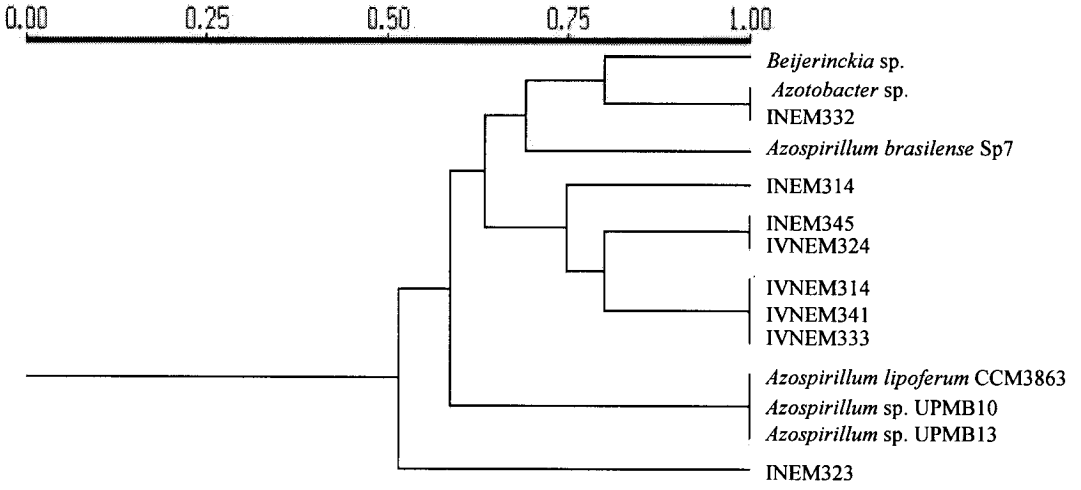

Figure 35. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North Eastern region from foot hill of mountain area

3.2.4 nif D PCR of free-living nitrogen fixing bacteria from rice in rotation with other crops area

Dendrogram of bacterial isolate in the North region found that strain in rainy season and dry strain as INCR410, INCR36 and IVCR34 were closely related to *Azospirillum brasilenese* Sp7. The similar strains in dry season as IVNCR15 and IVNCR43 were similar or closely related to IVNCR22 strain in dry season (Fig. 36). Dendrogram of isolate samples in the Central region indicated that IVCCR14 strain in dry season closely related to *Azospirillum brasilenese* Sp7 and *Beijerinckia* sp.. Bacterial strains as ICCR16 in rainy season and IVCCR31 in dry season were nearly related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirilum* sp. UPMB13. The results were indicated the similar strain in rainy and dry season as ICCR26, ICCR38 and IVCCR21. Figure 37, similar strains in both seasons such as ICCR41, IVCCR41 and the dominant native strain. Dendrogram in the North Eastern in season found that INECR11 strain in rainy season was closely related to *Azotobacter* sp. and the similar strains in both seasons as INECR22, INECR44 and IVNECR12 were closely related to *Azotobacter* sp. and *Beijerinckia* sp.. While the same strains in both seasons such as INECR37 and IVNECR44 were closely related to *Azotobacter* sp. and *Beijerinckia* sp.. Bacterial isolated strain in dry season as IVNECR33 in dry season was nearly related to *Azospirillum brasilenese* Sp7 while IVNECR22 strain in dry season was belong in an another different group (Fig. 38).

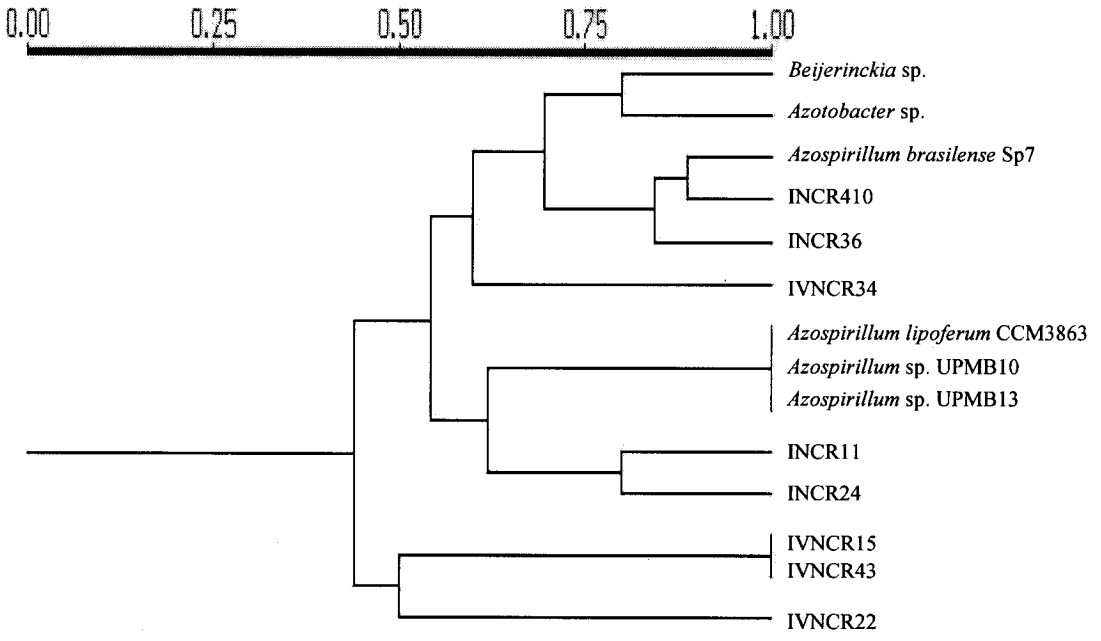

Figure 336. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North region from rice in rotation with other crops area.

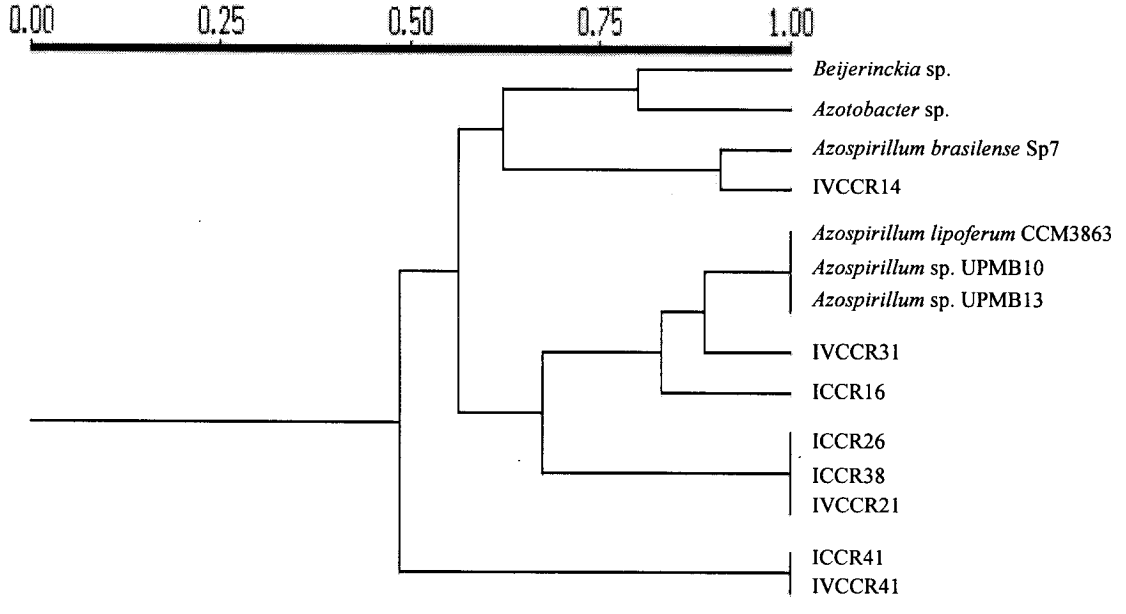

Figure 37. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the Central region from rice in rotation with other crops area.

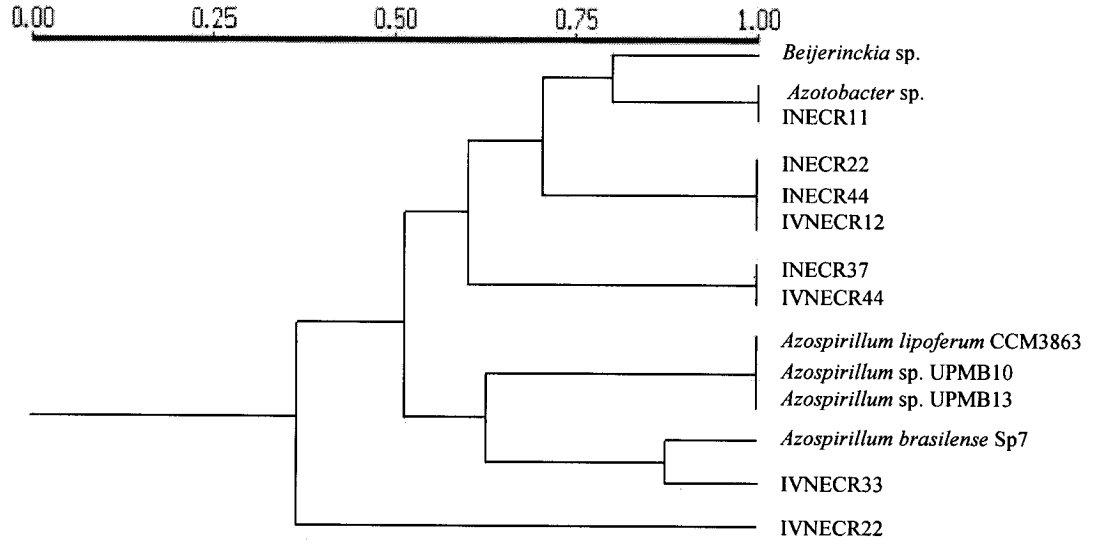

Figure 38. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North Eastern region from rice in rotation with other crops area

3.2.5 nif D PCR of free-living nitrogen fixing bacteria from rice cultivation area

Dendrogram generated to showed the similar strains in rainy such as IVNR14 and IVNR26. Morever, this also indicated similar strains in rainy and dry season as INR33, INR43, IVNR44 and IVNR32. However, bacterial isolated strains in rainy season as INR12 was closely related to INR33, INR43, IVNR44 and IVNR31. On the other hand, the similar strains in rainy season as INR25 was in another different group (Fig. 39). Dendrogram of the Central region indicated the similar strain in rainy season as ICR44 and *Beijerinckia* sp.. The results were shown the similar strains in both season as ICR12, ICR24, ICR35 and IVCR41 while the similar strains in dry season were IVCR31 and IVCR33. Strain in dry season as IVCR12 was displayed closely related to *Azospillirum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 (Fig.40). Dendrogram from the North Eastern found that similar strains in both season as INER15, INER24 and IVNER43 were indicated closely related to *Beijerinckia* sp. Bacterial strain in both seasons as IVNER11 and INER46 were found closely related to *Azotobacter* sp. and *Azospirillum brasilenese* Sp7. Strains in dry season as IVNER21 and IVNER31 were found nearly related to *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 (Fig. 41).

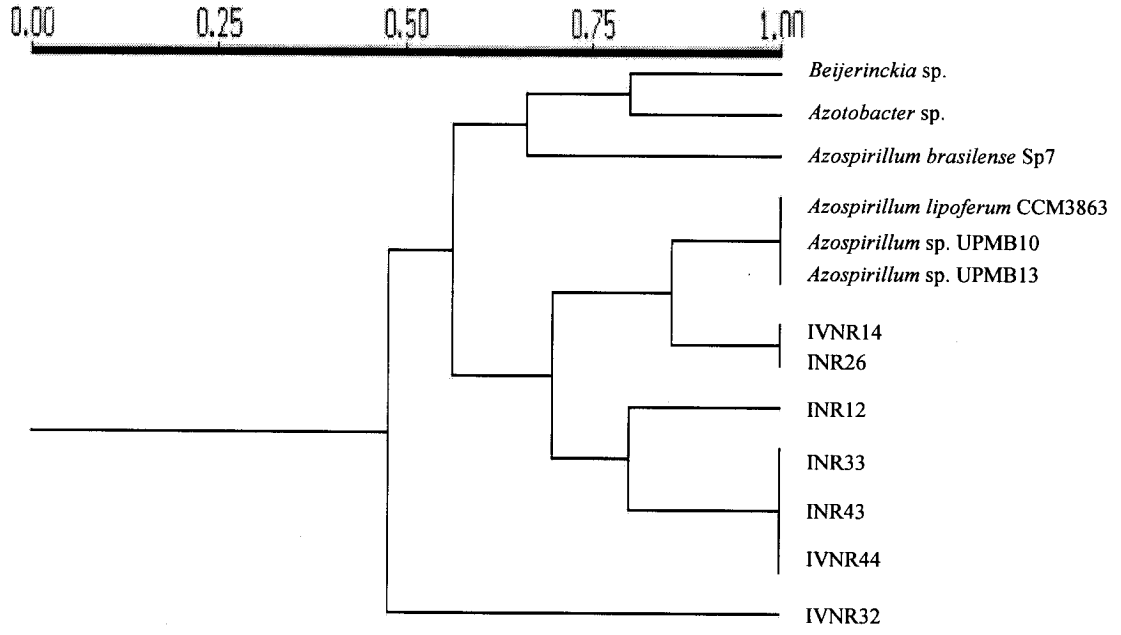

Figure 39. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North region from rice cultivation area.

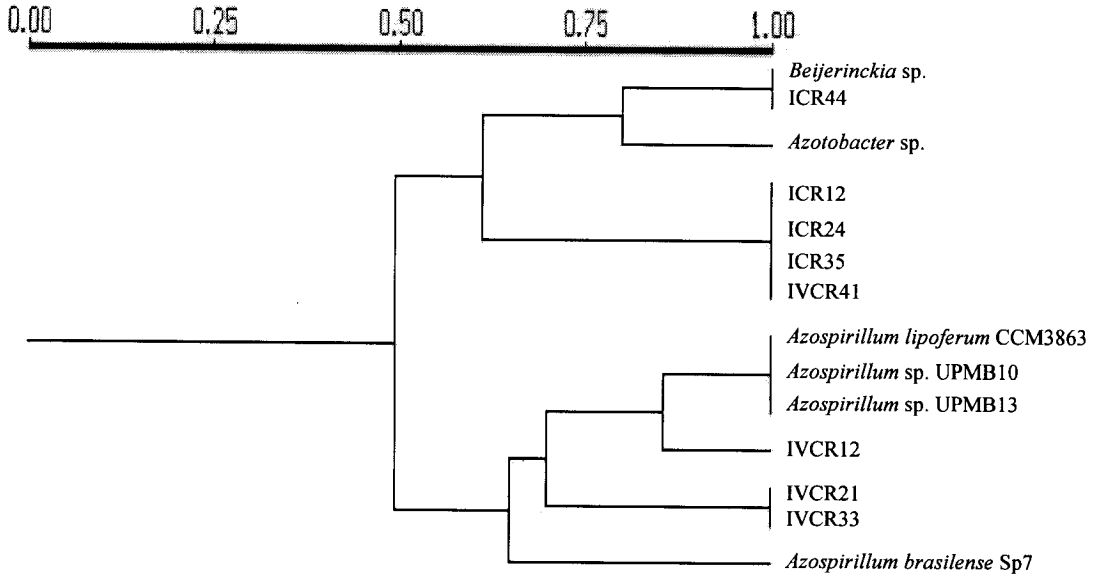

Figure 40. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the Central region from rice cultivation area.

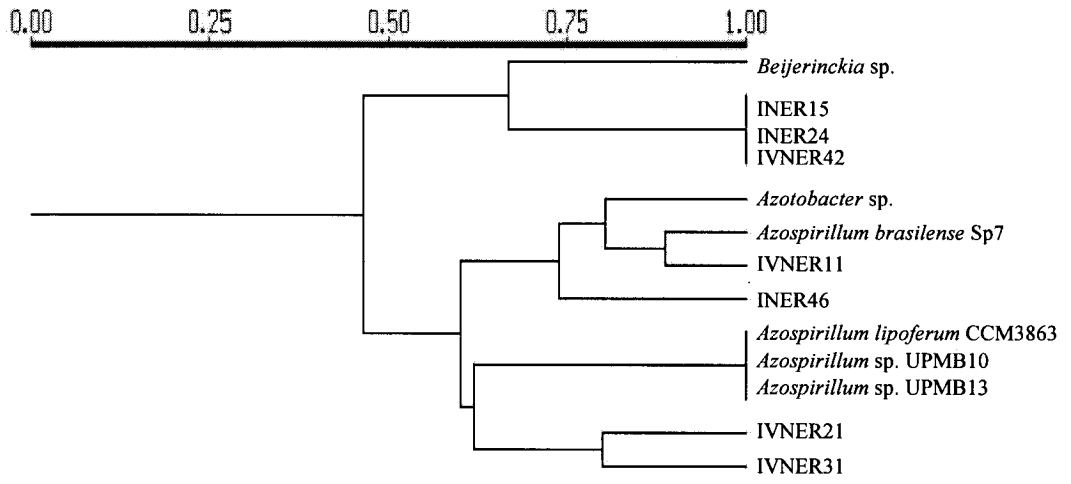

Figure 41. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North Eastern region from the rice cultivation area

3.2.6 nif D PCR of free-living nitrogen fixing bacteria from the field crop cultivation area

Dendrogram in the North region of bacterial strains were found that similar strains in dry season as IVNC13 and IVNC31 were closely related to *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13, *Beijerinckia* sp and *Azotobacter* sp.. Similar strains in dry season and rainy season as INC31 and IVNC24 were closely related to *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13, *Beijerinckia* sp and *Azotobacter* sp.. Bacterial isolated strains in rainy season as INC29 and INC15 were found closely related to *Azospirillum brasilenese* Sp7. Strains in both seasons as INC45 and IVNC41 were closely related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 (Fig. 42). Drodogram in the Central region was found that bacterial strains showed similar strain in the dry season as IVCC13, IVCC24 and IVCC32. Bacterial strains in rainy and dry season as IVCC45, ICC11, ICC42 and ICC34 were found that closely related to *Beijerinckia* sp., *Azotobacter* sp and *Azospirillum brasilenese* Sp7. Strain in rainy season as ICC25 was nearly related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 (Fig. 43). Dendogram in the North Eastern found that similar strains in dry season as IVNEC12, IVNEC22, IVNEC33 and IVNEC42 and strain in rainy season as INEC14 were closely related to *Beijerinckia* sp and *Azotobacter* sp. Bacterial isolates in rainy season as INEC23, INEC33 and INEC49 were closely related to *Azospirillum brasilense* Sp7 (Fig. 44).

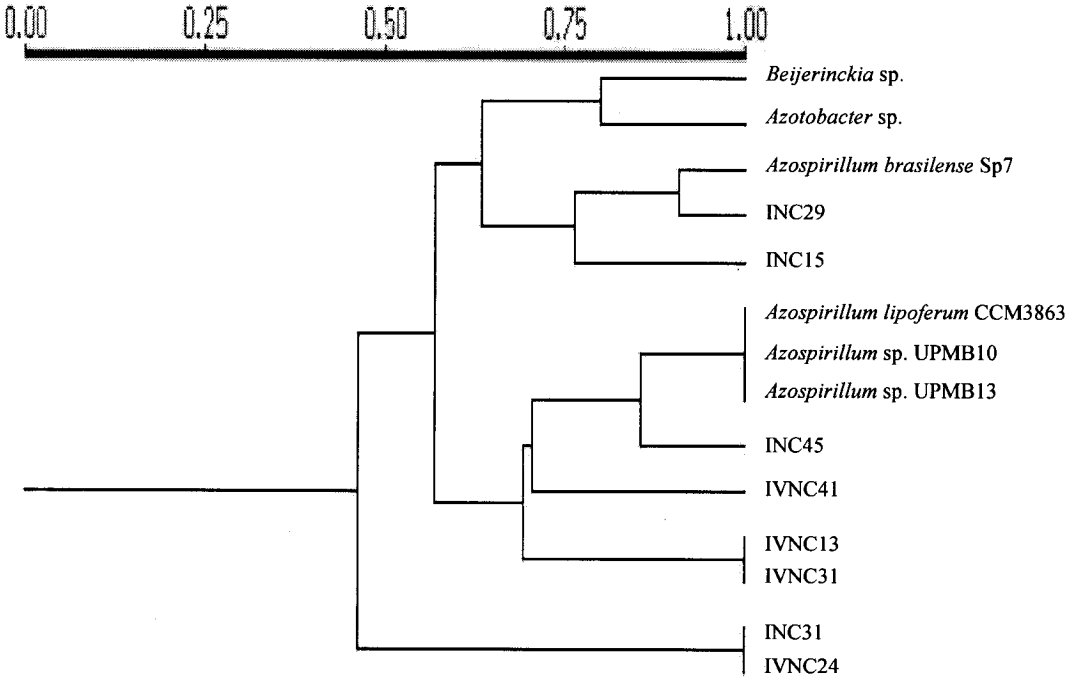

Figure 42. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North region from the field crop cultivation area

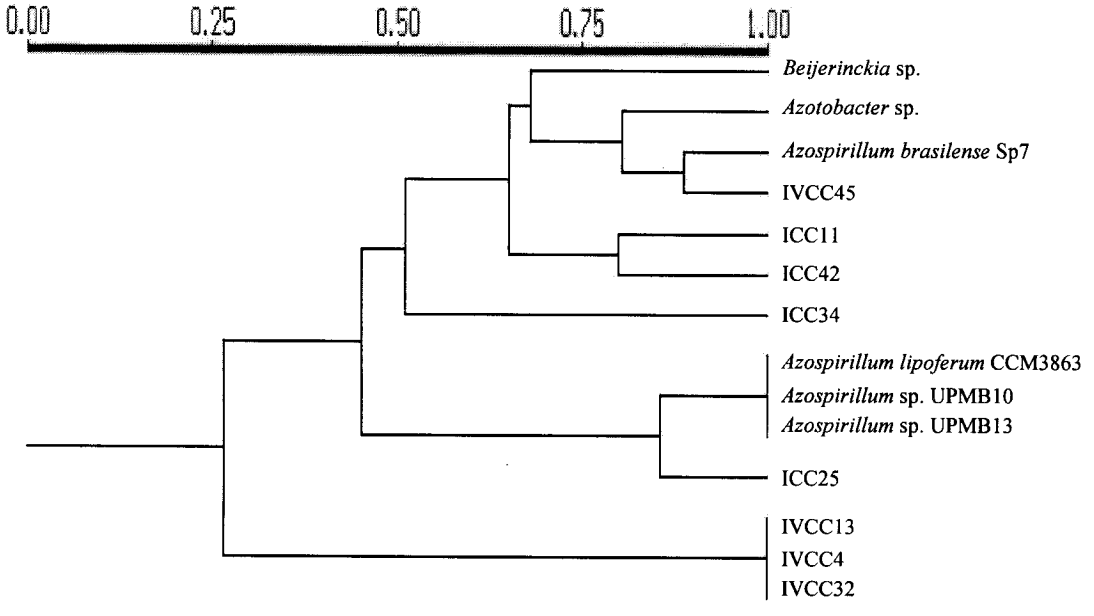

Figure 43. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the Central region from the field crop cultivation area

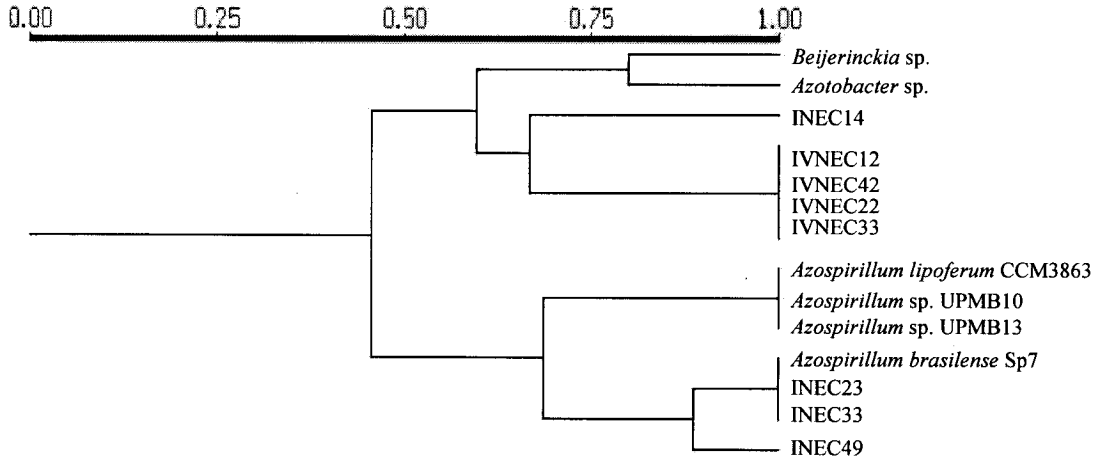

Figure 44. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North Eastern region from the field crop cultivation area

3.2.7 nif D - PCR of free-living nitrogen fixing bacteria from uncultivated area.

Dendrogram in the North region of isolated sample in the dry season as IVNF11 was similar to *Beijerinckia* sp. and INF32 isolated sample in rainy season was similar to *Azotobacter* sp.. Free-living nitrogen fixing bacterial strains found similar strains in both season as INF25 and IVNF31 were closely related to *Beijerinckia* sp. and *Azotobacter* sp.. Similarity of bacterial isolates in dry season as IVNF24 and IVNF41 and strains in rainy season as INF16 and INF47 were displayed closely related to *Azospirillum brasilenese* Sp7, *A.lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 (Fig. 45). Dendrogram from the Central region showed the similar strains in both seasons as ICF15, ICF24, ICF39 and IVCF32. Isolate from dry season as IVCF42 showed closely related to *Beijerinckia* sp., *Azotobacter* sp. and *Azospirillum brasilenese* Sp7. Bacterial strain in dry season as IVCF13 was found closely related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. Isolated strain in both seasons as ICF43 and IVCF24 were belong in an another different group (Fig. 46). The phylogenetic tree in the North Eastern of isolate in rainy season as INEF33 strain was similar to *Beijerinckia* sp., INEF44 strain in rainy season strain was similar to *Azotobacter* sp. and strain in dry season IVNEF12 was similar to *Azospirillum brasilenese* Sp7. For bacterial isolates were shown similarity strain among free-living nitrogen fixing bacteria group and the dominant native strains as INEF27 and IVNEF24 were found. The bacterial strains as INEF31, INEF27, IVNEF24 and INEF47 in rainy season and dry season was found closely related to

Beijerinckia sp.and *Azotobacter* sp. and strain as IVNEF12 in dry season was shown closely related to *Azospirillum brasilenese* Sp7 (Fig. 47).

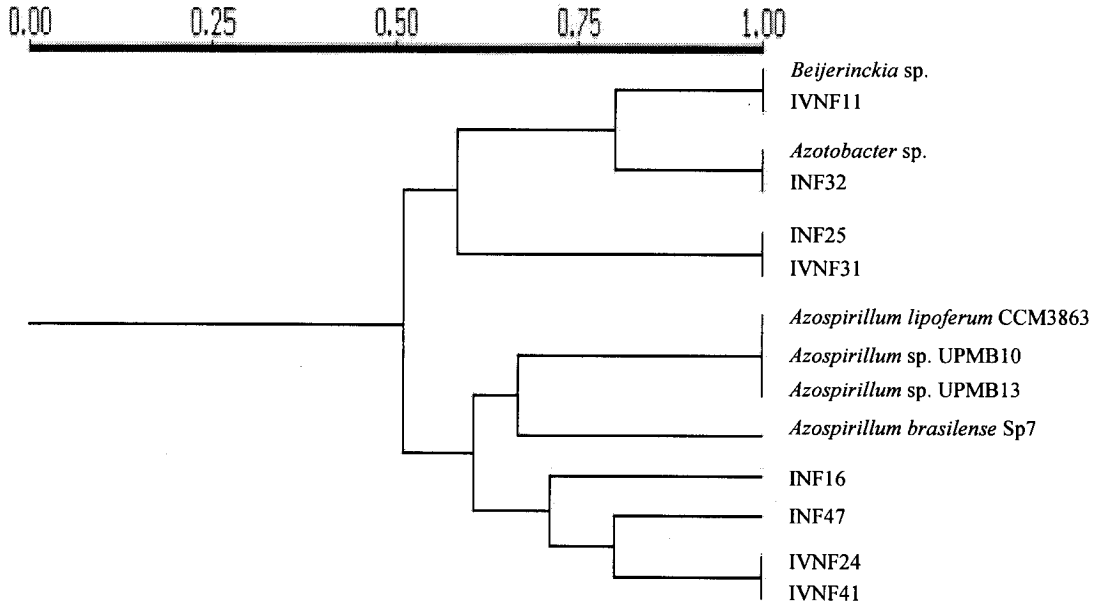

Figure 45. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North region from uncultivated area

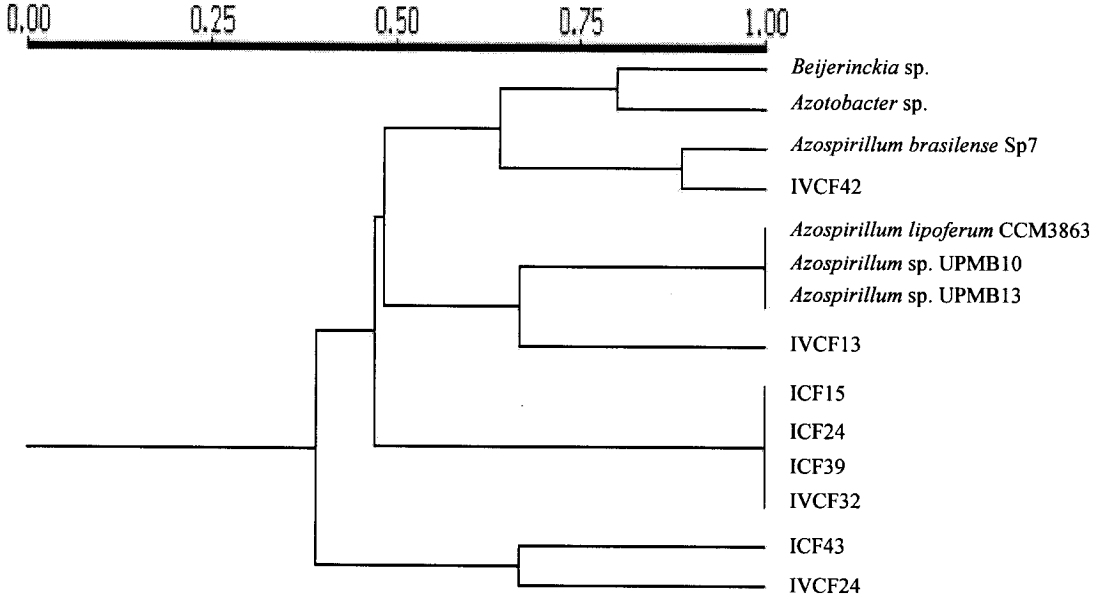

Figure 46. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the Central region from uncultivated area

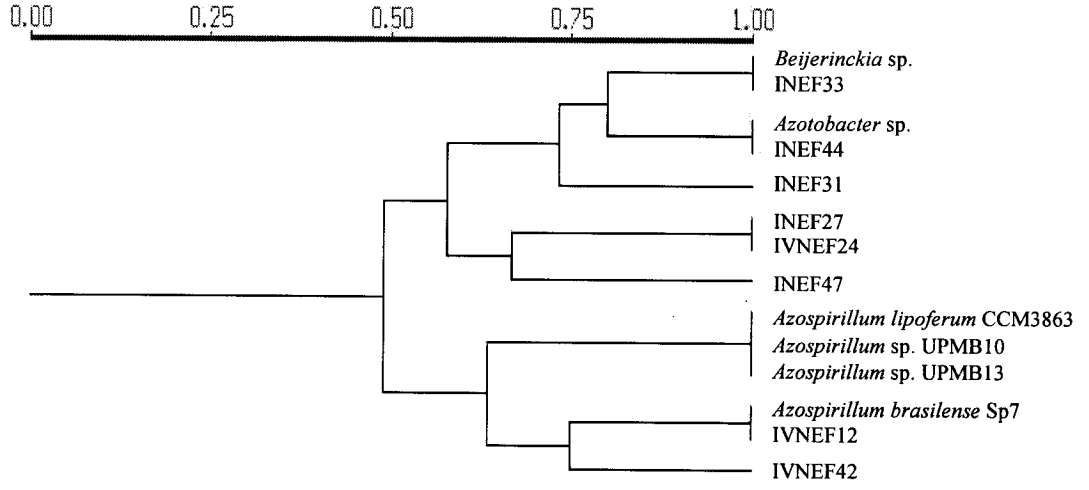

Figure 47. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North Eastern region from uncultivated area

3.2.8 nifD - PCR-of free-living nitrogen fixing bacteria from intensive agriculture production using high rate of pesticides and fertilizers area

Dendrogram of bacterial isolates in the North region were found similar strain as IND13 and IND24 in rainy season and IND13, IND24 and VND26 in both seasons were closely related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. Strain in rainy season as IND39 was in another different group. Isolates was indicated that similar strain in dry season as VND13 to *Beijerinckia* sp.. The bacterial isolate in dry season such as VND31was found closely related to *Azospirillum brasilenese* Sp7 (Fig. 48). While dendrogram of isolated samples in Central region were found similar strains in rainy season as ICD21 and ICD31 were closely related to *Beijerinckia* sp.. Isolated strain in dry season as VCD31 was found closely related to *Azospirillum brasilenese* Sp7, *A.lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. Strain in dry season such as VCD11 was nearly related to *Azospirillum brasilenese* Sp7. Bacterial strains in both seasons as ICD13 and VCD21 were in another different group (Fig. 49). Dendrogram of isolates in the North Eastern region were displayed that bacterial strains in both seasons as INED17, VNED11 and VNED37 were nearly related to *Beijerinckia* sp. and *Azotobacter* sp. Bacterial isolates from rainy season and dry season as INED23, INCD33 were found closely related to *Azospirillum brasilenese* Sp7, *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 (Fig. 50).

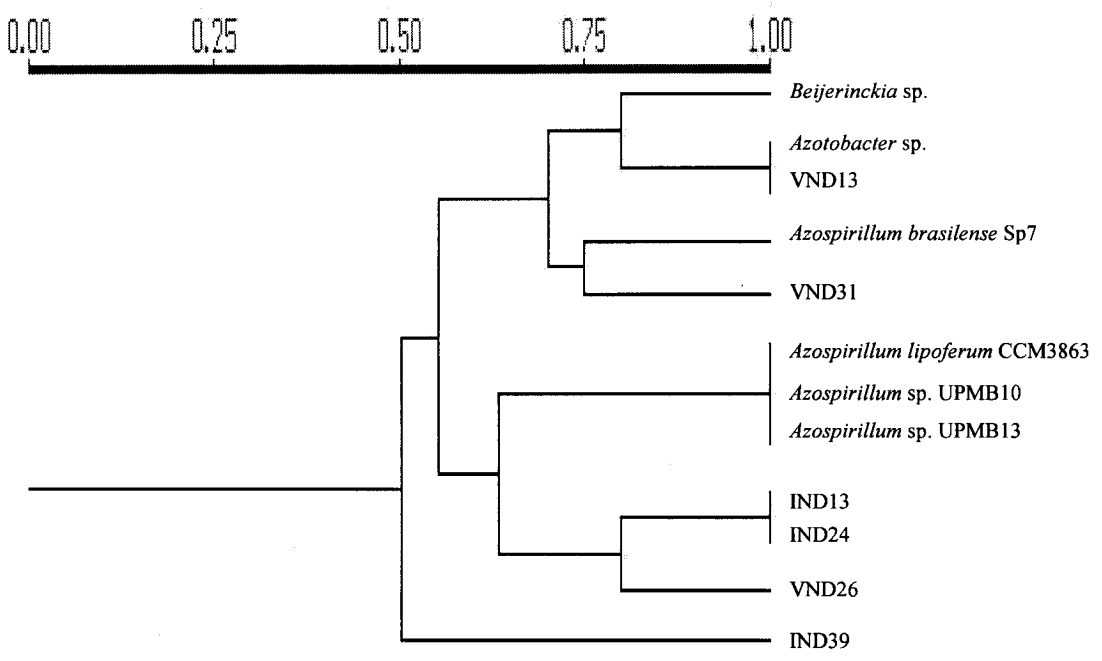

Figure 48. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North region from intensive agriculture production using high rate of pesticides and fertilizers area

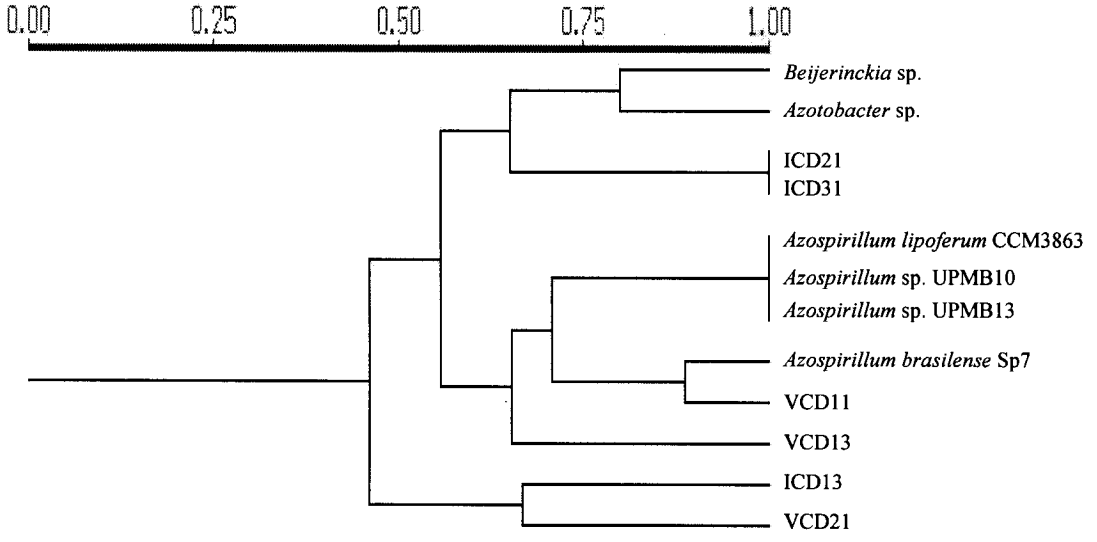

Figure 49. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the Central region from intensive agriculture production using high rate of pesticides and fertilizers area

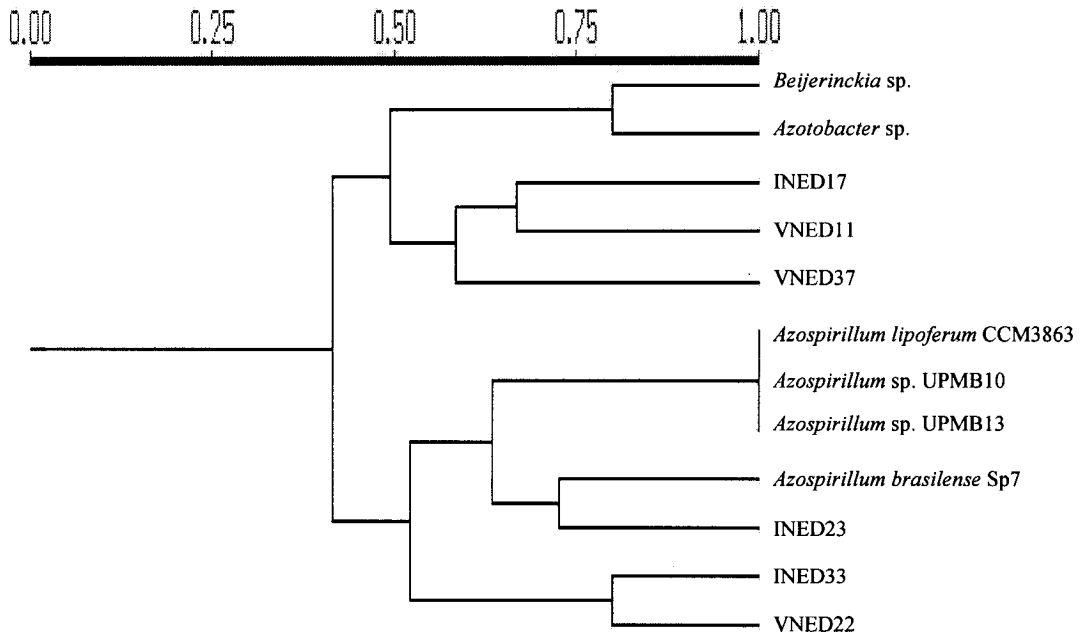

Figure 50. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North Eastern region from intensive agriculture production using high rate of pesticides and fertilizers area

3.2.9 nif D - PCR of free-living nitrogen fixing bacteria from undisturbed forest area

Dendrogram from the North region of strains in both seasons as INA19, VNA13 and VNA23 were closely related to *Azotobacter* sp. and *Beijerinckia* sp. INA19 and VNA13 were the dominant native strain. The bacterial strain in rainy season as INA23 was nearly related to *Azospirillum brasilense Sp7, A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 (Fig. 51). Figure 52, the dendrogram in the Central region of free-living nitrogen fixing bacteria in both seasons as ICA23, ICA16, VCA22 and VCA12 were closely related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. Dendrogram of isolated samples in the North Eastern in rainy season were found that similar strain in rainy strain as INER17, INER27, INEA17 and INEA27 strains in rainy season were found closely related to *Beijerinckia* sp. and *Azotobecter* sp.. Strain in dry season as VNEA21 was same to *Azotobacter* sp.. For isolated strain in dry season as VNEA11 was belong in an another different group (Fig. 53).

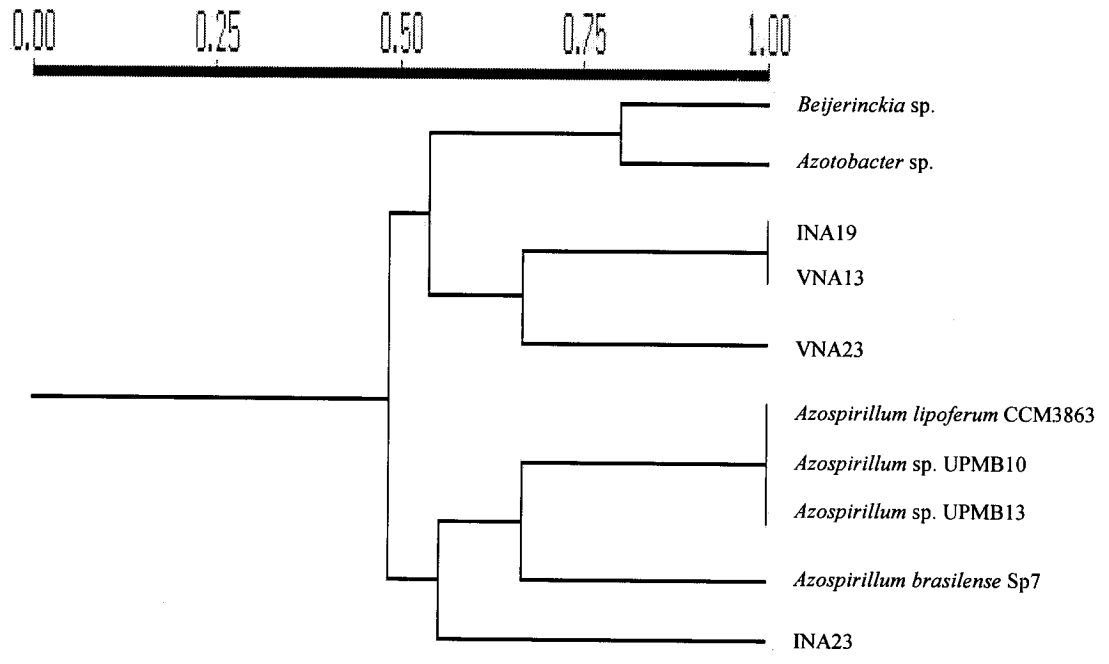

Figure 51. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North region from undisturb forest area

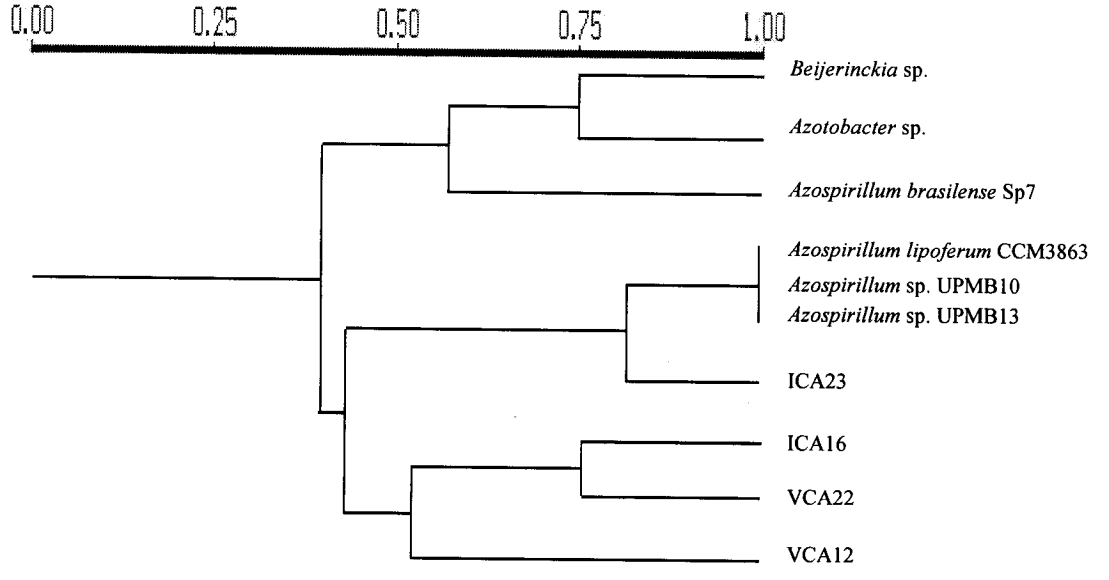

Figure 52. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the Central region from undisturb forest area

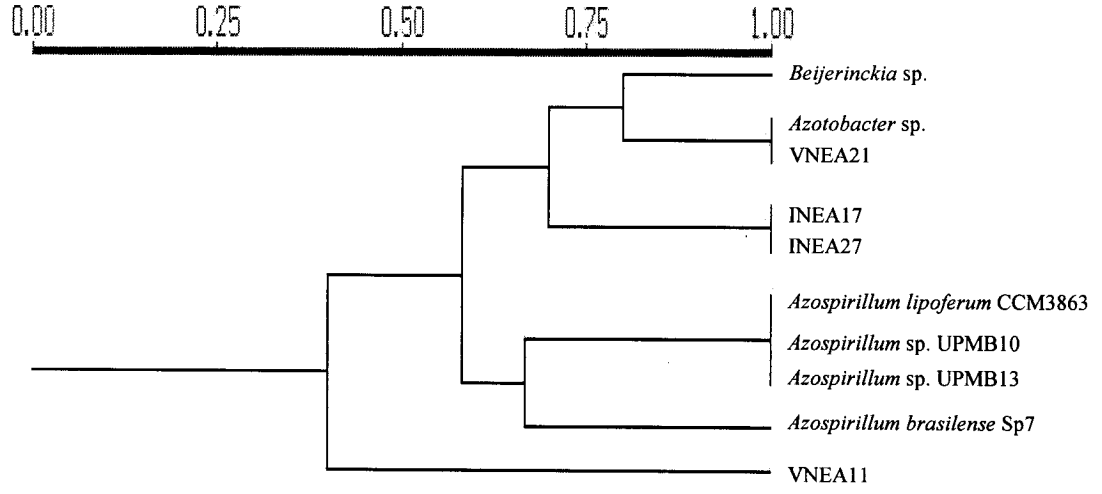

Figure 53. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North Eastern region from undisturb forest area

3.2.10 nif D –PCR of free-living nitrogen fixing bacteria from forest clearance for crop cultivation for 1-2 years

The dendrogram of isolates in the North region in rainy season were found nearly related to *Beijerincka* sp.. Similar isolated strains in dry season as VNB14 and VNB21 were closely related to *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 (Fig. 54). Dendrogram of bacterial isolates in the Central found that similar strains as ICB12 and ICB27 in rainy season and VCB strain in dry season were nearly related to *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. The strain in dry season as VCB11 was belong in an another different group (Fig. 55). For the dendrogram of isolated samples in the North Eastern region showed that similar strains in dry and rainy season as INEB21 and VNEB21 and the dominant native strain and INEB11 strain in rainy season were obtained closely related to *Beijerincka* sp. and *Azotobacter* sp. For isolated strain in dry season as VNEB14 was shown nearly related to *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 (Fig. 56).

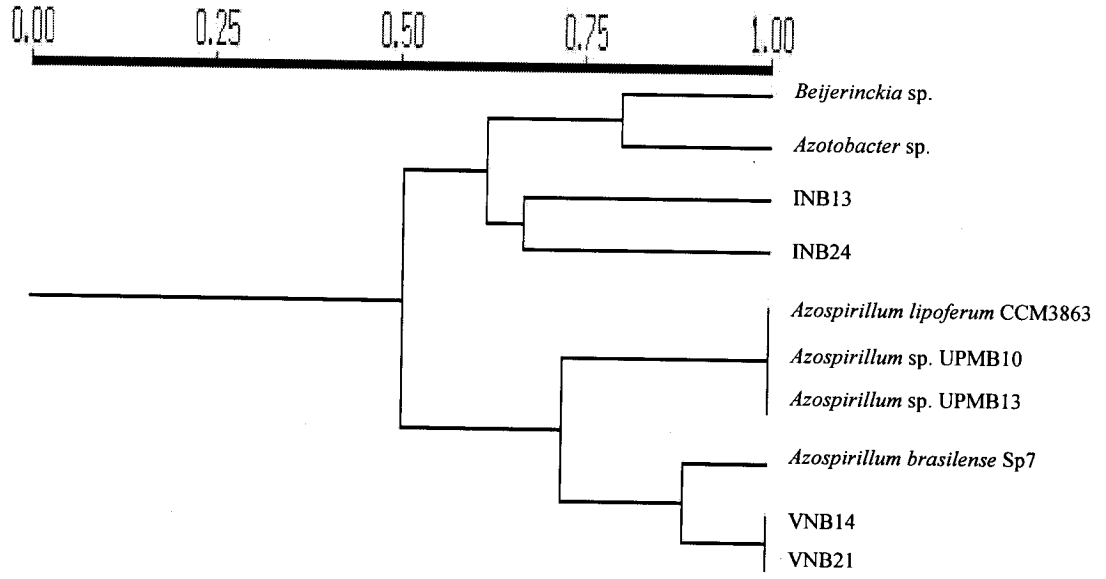

Figure 54. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and hot season in the North region from forest clearance for crop cultivation for 1-2 years area.

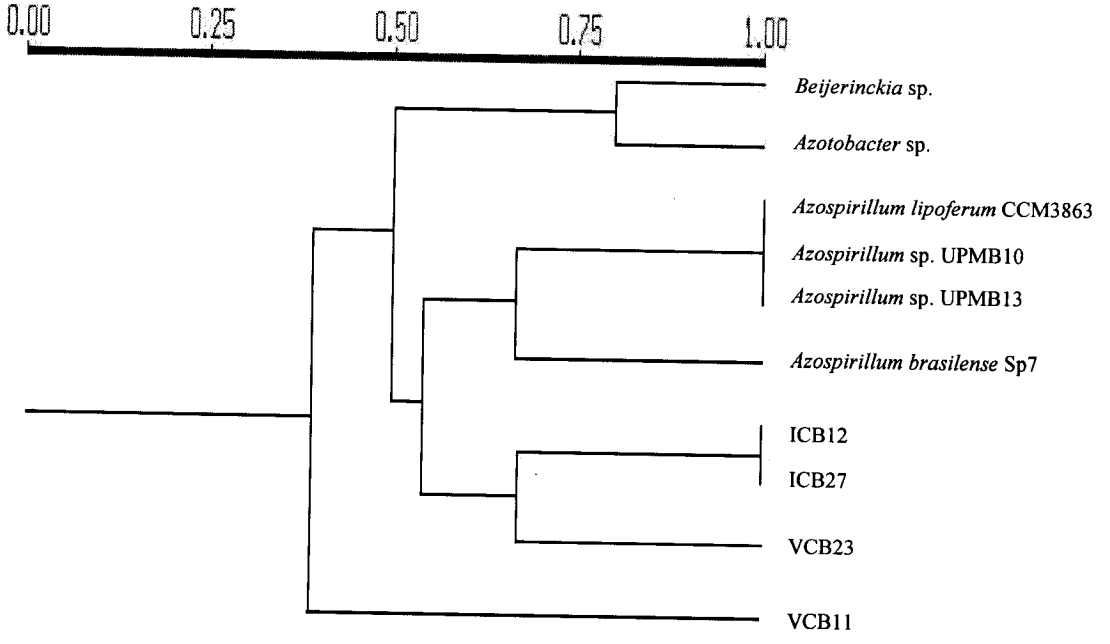

Figure 55. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and hot season in the Central region from forest clearance for crop cultivation for 1-2 years area.

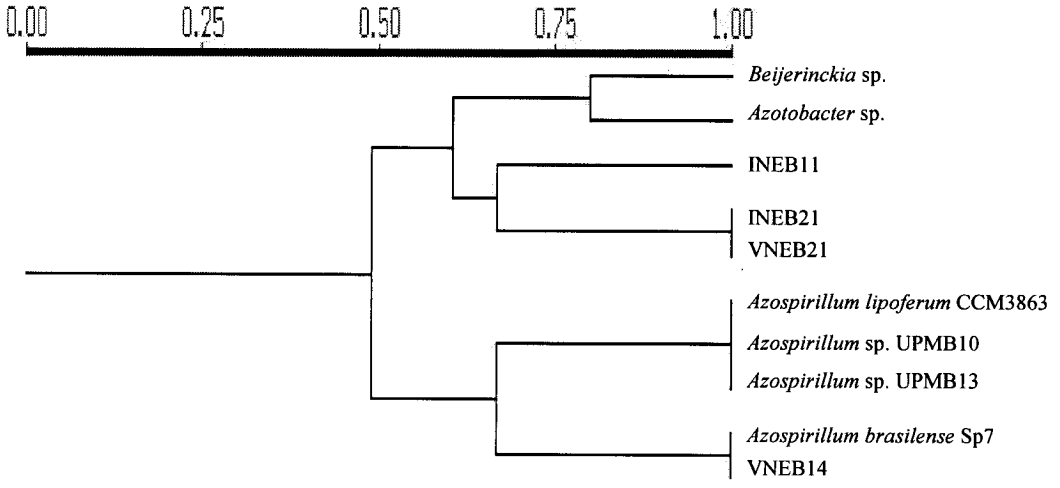

Figure 56. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North Eastern region from forest clearance for crop cultivation for 1-2 years area

3.2.11 nif D - PCR of free-living nitrogen fixing bacteria from forest clearance for crop cultivation for 3 years area

Dendrogram of bacterial isolates in the North were shown similar stains as INC*28 and VNC*24 in both season and the dominant native strain. They were displayed closed related to *Azospirillum brasilenese* Sp7, *A.lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. The bacterial isolate in rainy season as INC*16 was belong in an another different group (Fig. 57). Dendrogram in the Central region of strain in rainy season as ICC*13 was found nearly related to *Azospirillum brasilenese* Sp7, *Beijerinckia* sp. and *Azotobacter* sp.. Isolated strains in both seasons as ICC*25, VCC*11 and VCC*25 were obtained closely related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 (Fig. 58). Dendrogram in the North Eastern of bacterial strain in dry season was found closely related to *Beijerinckia* sp. and *Azotobacter* sp.. Bacterial strains in both season as INEC*16, INEC*23 and VNEC*11 were nearly related to *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 (Fig. 59).

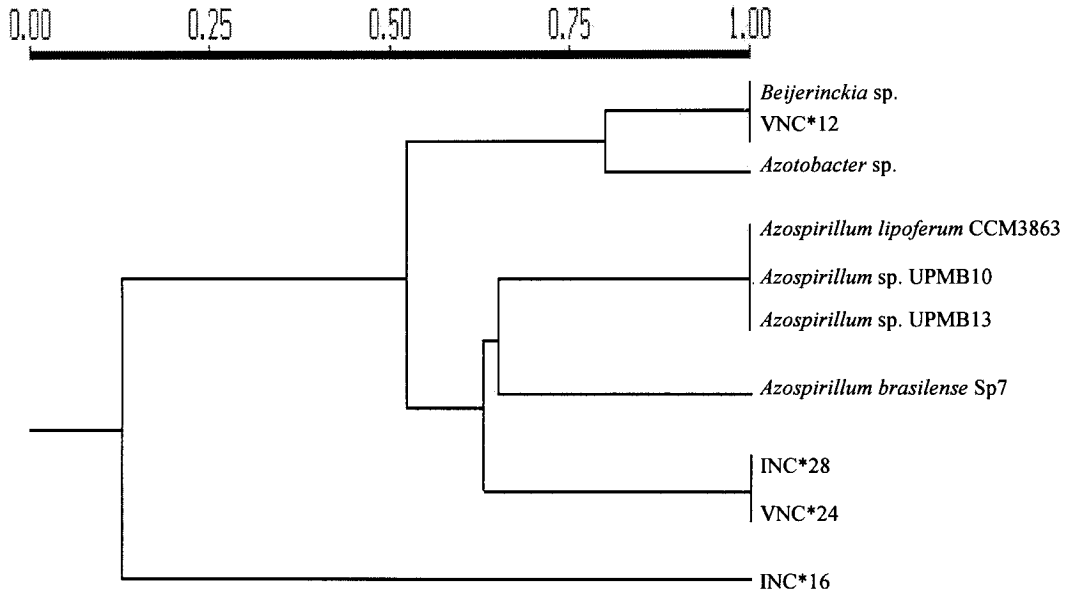

Figure 57. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North region from forest clearance for crop cultivation for 3 years area

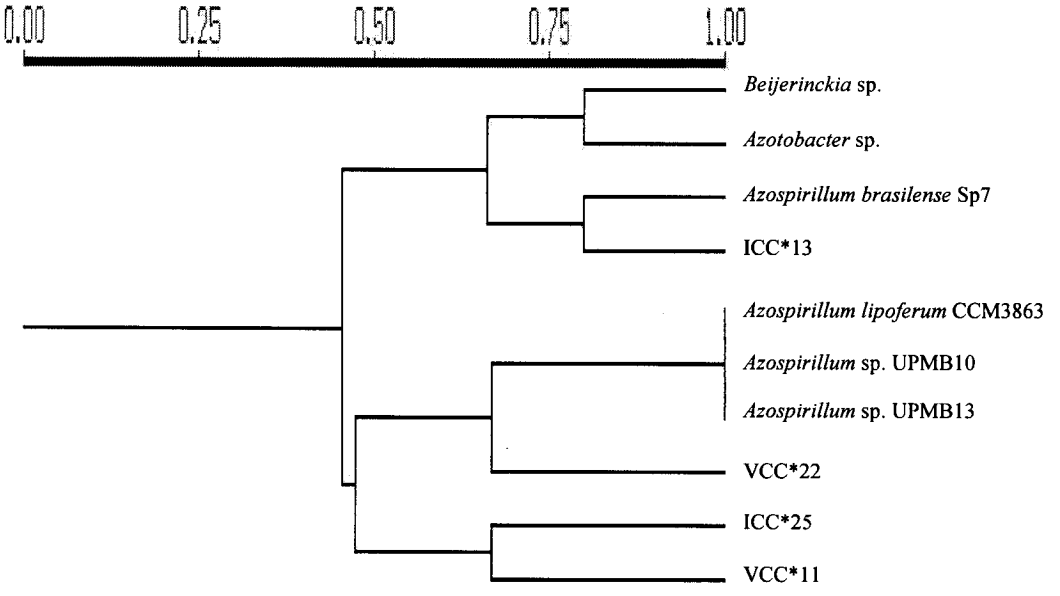

Figure 58. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the Central region from forest clearance for crop cultivation for 3 years area

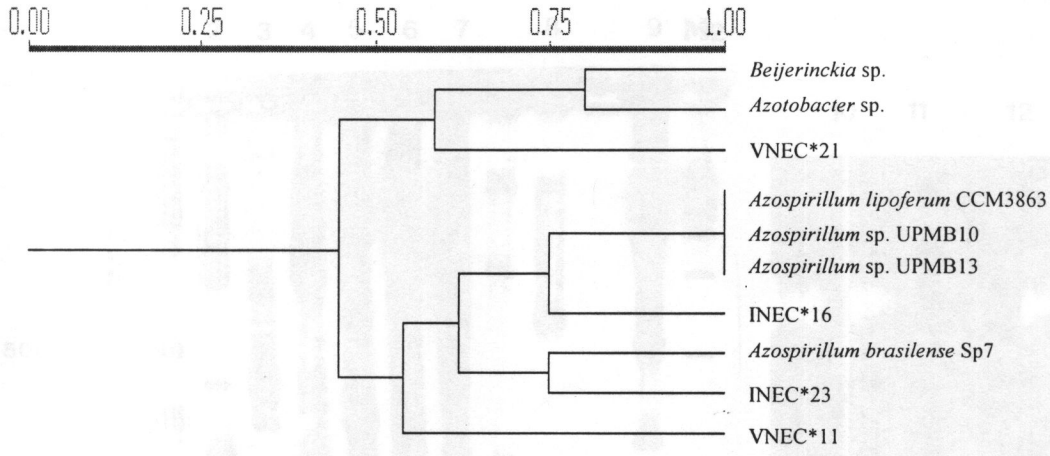

Figure 59. Dendrogram of free-living nitrogen fixing bacteria isolate compared between rainy season and dry season in the North Eastern region from forest clearance for crop cultivation for 3 years area

3.3 Enterobacterial repetitive intergenic consensus (ERIC) primer profile analysis

To elucidate the diversification of free-living nitrogen fixing bacteria in each *nif* D-PCR product group, ERIC primer was conducted since this primer could be detected in a large variety of eubacteria that it was gram-negative bacteria. ERIC-PCR product patterns of bacterial strains were shown in Figure 60 to 85.

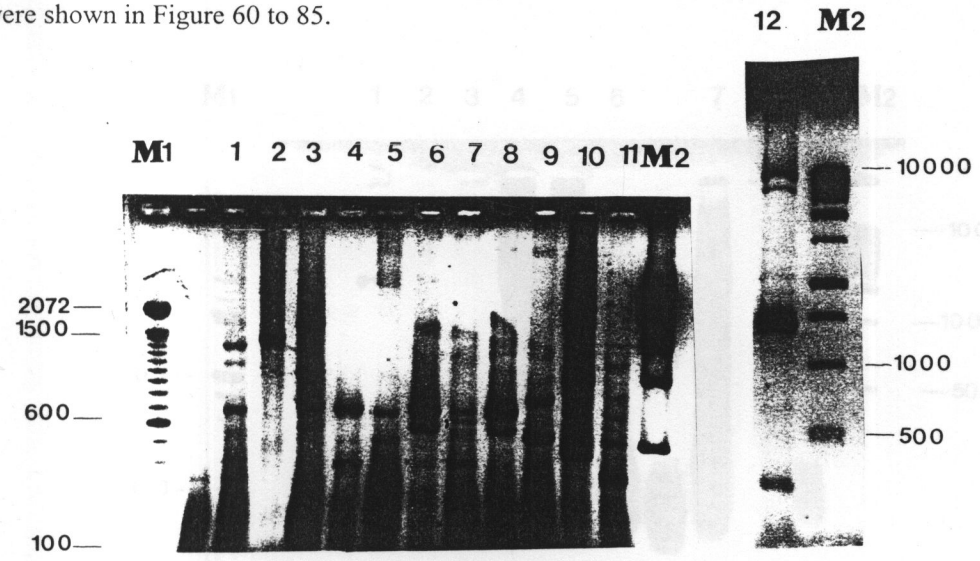

Figure 60. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker; Lane M2, 1Kbp. Ladder marker; Lane 1, INM125; Lane 2, INM133; Lane 3, INM145; Lane 4, ICM118; Lane 5, ICM123; Lane 6, ICM137; Lane 7, ICM147; Lane 8, INEM114; Lane 9, INEM123; Lane 10, INEM133; Lane 11, INEM143 and Lane 12, INM111

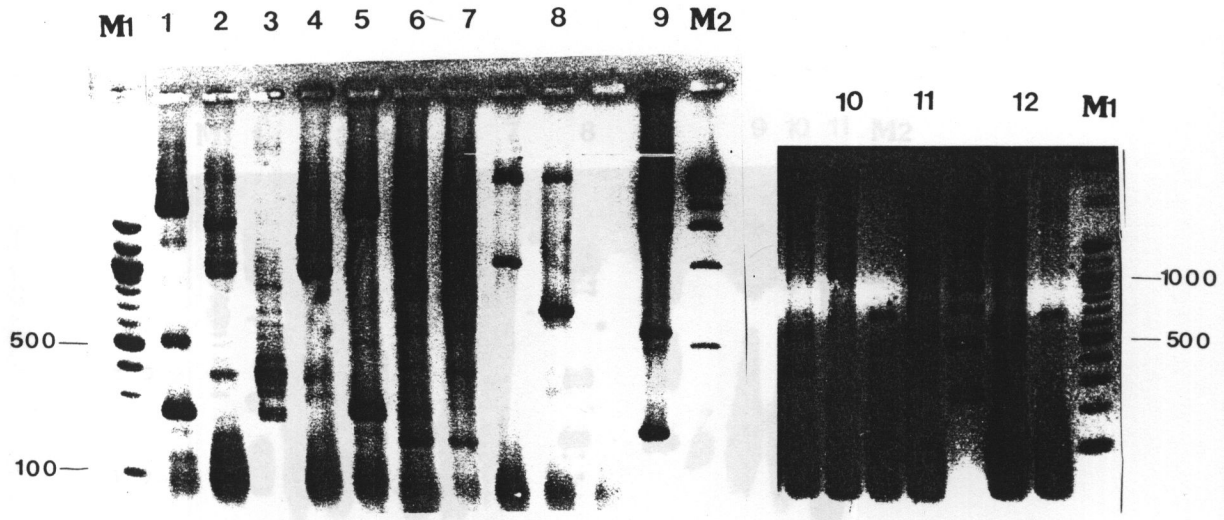

Figure 61. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker; Lane M2, 1 Kbp. Ladder marker; Lane 1, IVNM125; Lane 2, IVNM134; Lane 3, IVNM142; Lane 4, IVCM113; Lane 5, IVCM123; Lane 6, IVCM131; Lane 7, IVCM144; Lane 8, IVNEM121; Lane 9, IVNEM141; Lane 10, IVNEM112; Lane 11, IVNEM133 and Lane 12, INED23

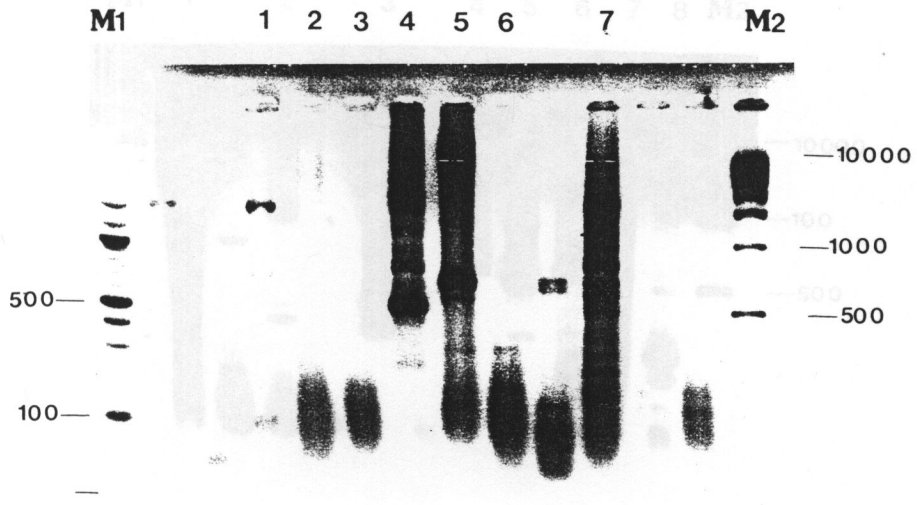

Figure 62. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker; Lane M2, 1 Kbp. Ladder marker; Lane 1, INM236; Lane 2, INM248; Lane 3, ICM219; Lane 4, ICM222; Lane 5, ICM235; Lane 6, ICM249 and Lane 7, INEM223

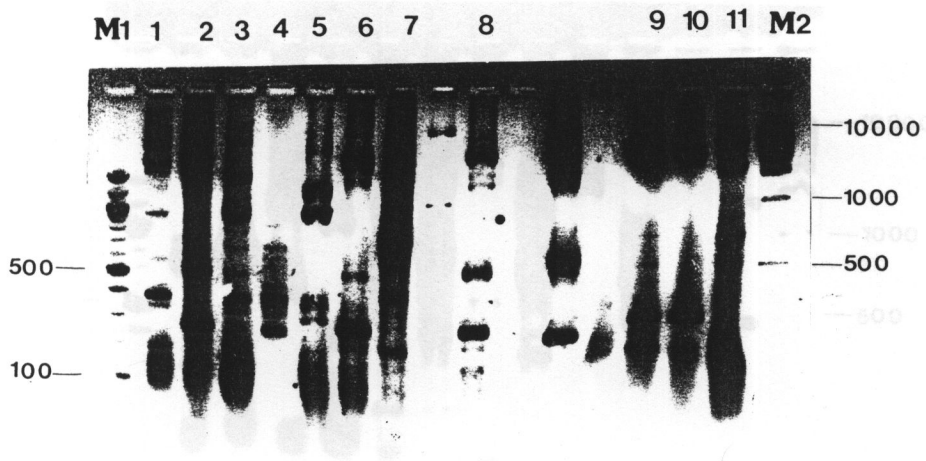

Figure 63. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker; Lane M2, 1 Kbp. Ladder marker; Lane 1, IVNM213; Lane 2, IVNM224; Lane 3, IVNM232; Lane 4, IVNM243; Lane 5, IVCM213; Lane 6, IVCM222; Lane 7, IVCM243; Lane 8, IVNEM241; Lane 9, INEC33; Lane 10, INEC48 and Lane 11, ICA16

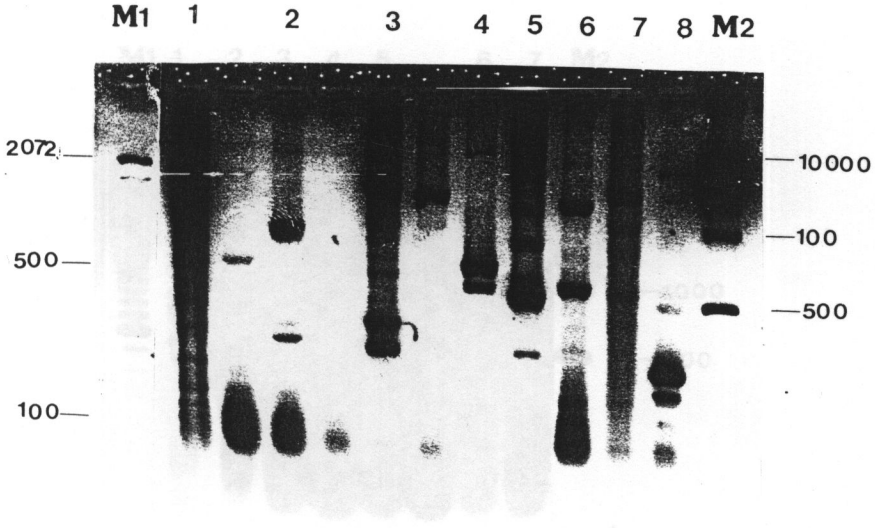

Figure 64. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, INM324; Lane 2, INM336; Lane 3, ICM321; Lane 4, ICM347; Lane 5, INEM314; Lane 6, INEM323; Lane 7, INEM332 and Lane 8, INEM345

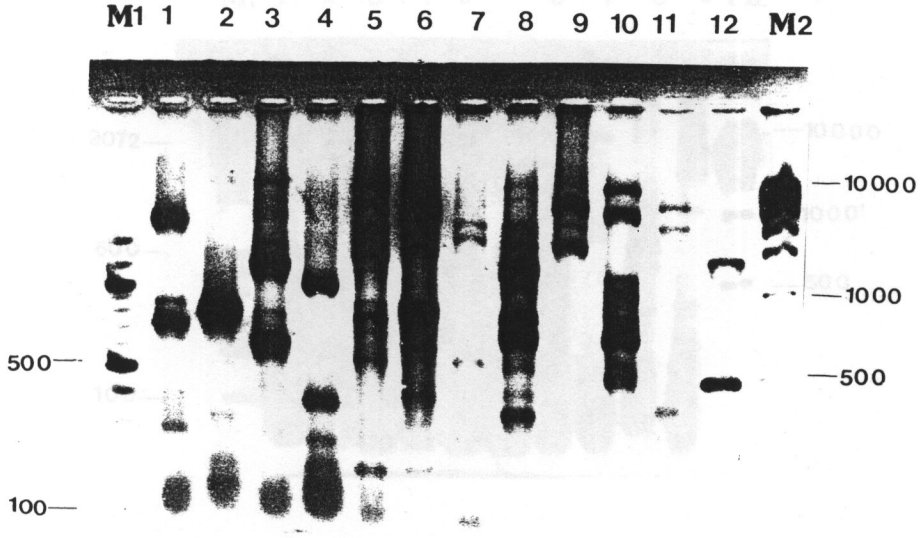

Figure 65. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, IVNM311; Lane 2, IVNM322; Lane 3, IVNM331; Lane 4, IVNM341; Lane 5, IVCM315; Lane 6, IVCM323; Lane 7, IVCM335; Lane 8, IVCM341; Lane 9, IVNEM314; Lane 10, IVNEM324; Lane 11, IVNEM333 and Lane 12, IVNEM341

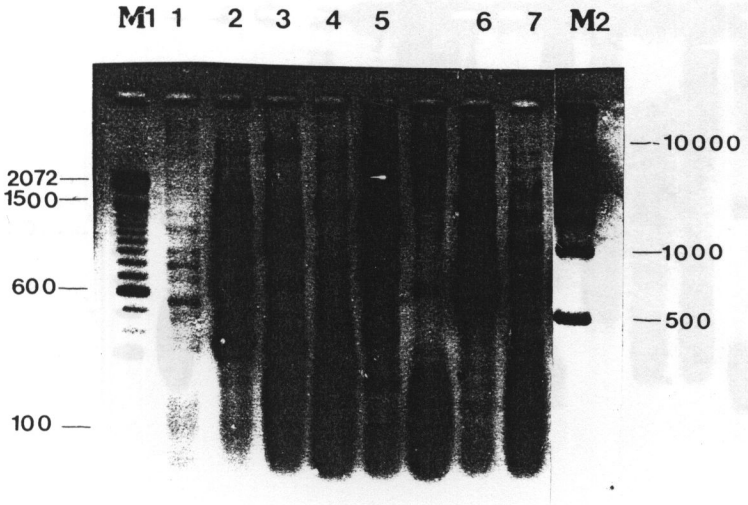

Figure 66. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, INCR11; Lane 2, INCR24; Lane 3, INCR36; Lane 4, INCR410; Lane 5, ICCR16; Lane 6, ICCR38 and Lane 7, ICCR48

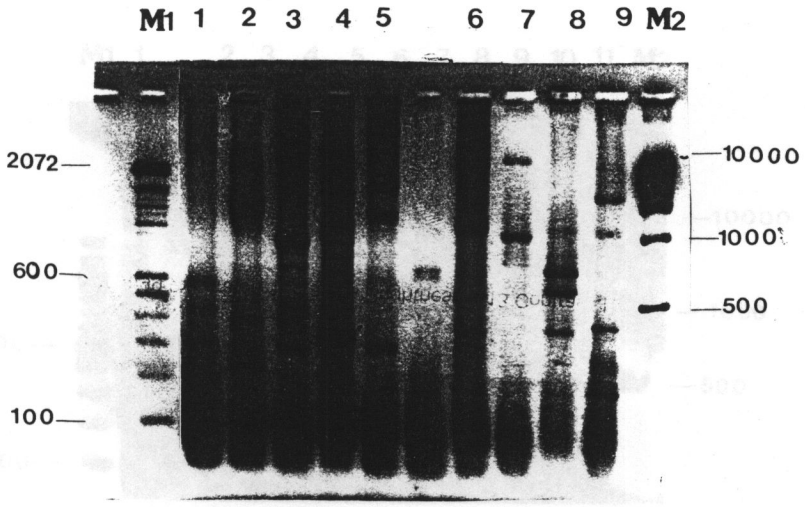

Figure 67. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, IVNCR34; Lane 2, IVNCR44; Lane 3, IVCCR14; Lane 4, IVNCCR21; Lane 5, IVCCR31; Lane 6, IVNECR12; Lane 7, IVNECR22; Lane 8, IVNECR33 and Lane 9, IVNECR44

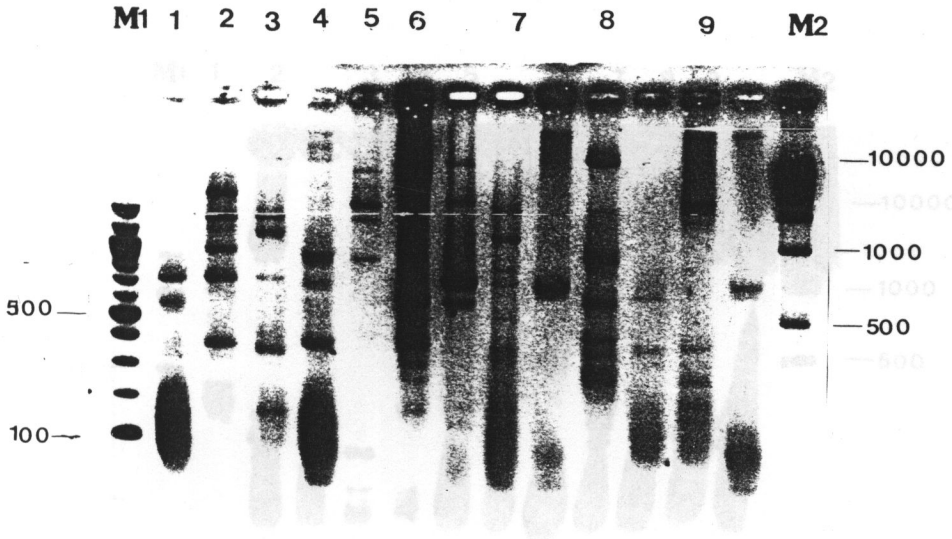

Figure 68. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, INR12; Lane 2, INR25; Lane 3, INR33; Lane 4, INR43; Lane 5, ICR12; Lane 6, ICR24; Lane 7, ICR44; Lane 8, INER24 and Lane 9, INER48

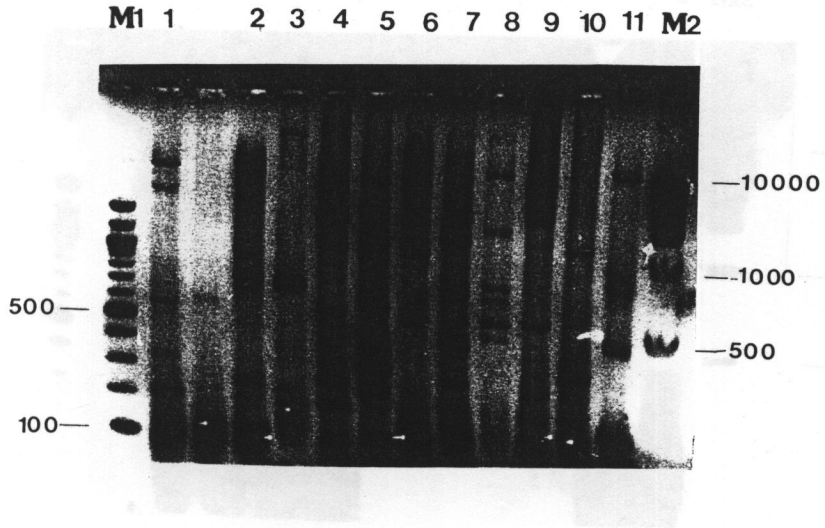

Figure 69. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker; Lane M2, 1 Kbp. Ladder marker; Lane 1, IVNR13; Lane 2, IVNR32; Lane 3, IVNR44; Lane 4, IVCR12; Lane 5, IVCR21; Lane 6, IVCR33; Lane 7, IVCR41; Lane 8, IVNER11; Lane 9, IVNER21; Lane 10, IVNER31 and Lane 11, IVNER42

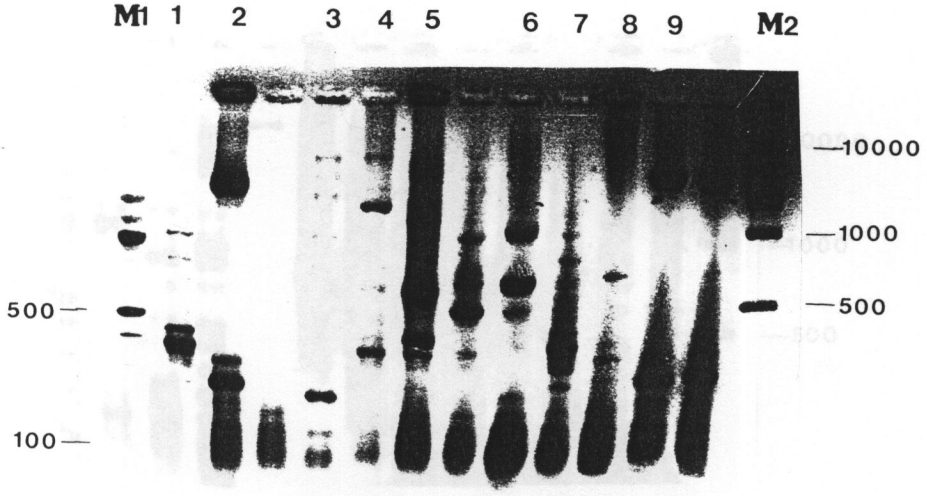

Figure 70. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, INC15; Lane 2, INC29; Lane 3, INC45; Lane 4, ICC11; Lane 5, ICC25; Lane 6, ICC42; Lane 7, INEC14; Lane 8, INEC23 and Lane 9, INEC33

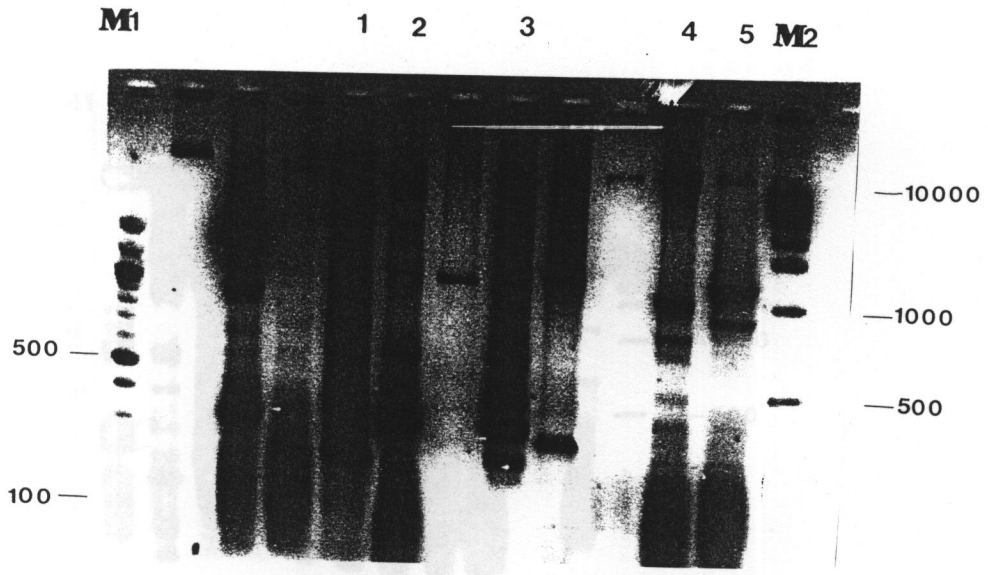

Figure 71. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, IVNC41; Lane 2, IVCC13; Lane 3, IVCC45; Lane 4, IVNEC33 and Lane 5, IVNEC42

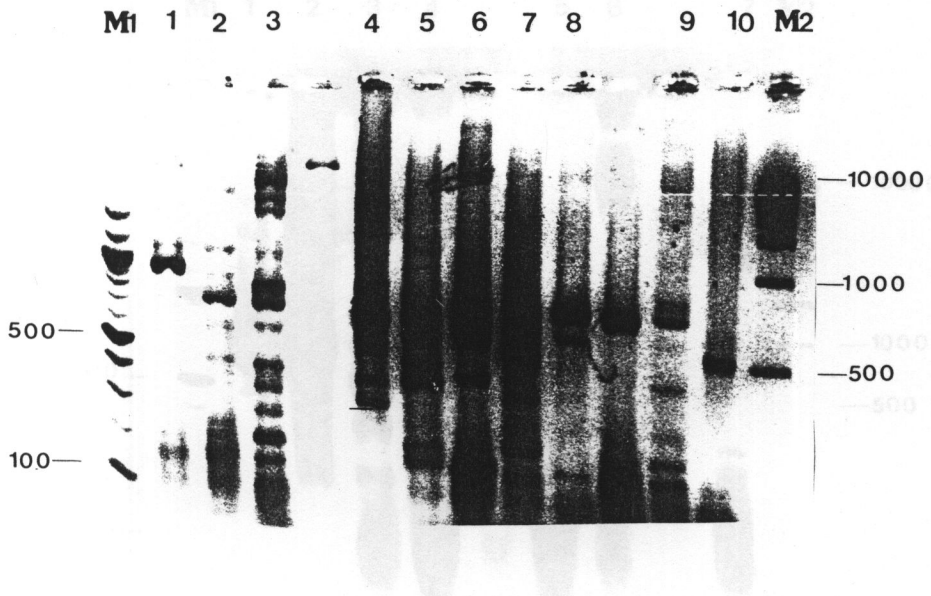

Figure 72. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, INF16; Lane 2, INF25; Lane 3, INF32; Lane 4, ICF15; Lane 5, ICF24; Lane 6, ICF39; Lane 7, ICF43; Lane 8, INEF14; Lane 9, INEF33 and Lane 10, INEF47

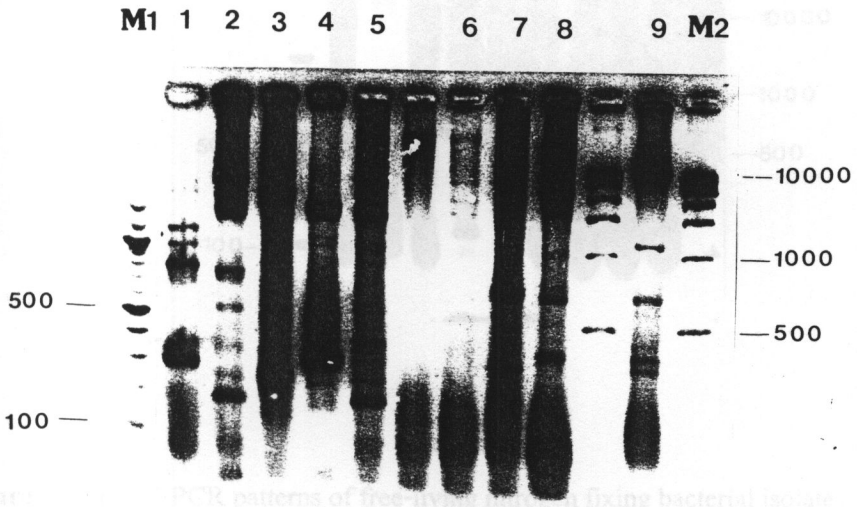

Figure 73. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp.Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, IVNF31; Lane 2, IVNF41; Lane 3, IVCF13; Lane 4, IVCF24; Lane 5, IVCF32; ; Lane 6, IVNEF12; Lane 7, IVNEF24; Lane 8, IVNEF31 and Lane 9, IVNEF42

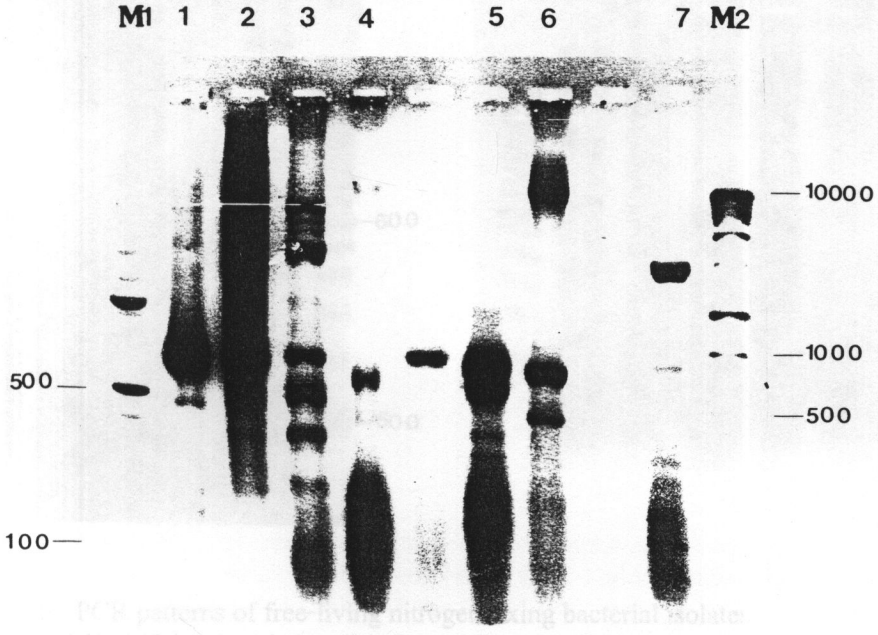

Figure 74. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, IND13; Lane 2, IND24; Lane 3, IND39; Lane 4, ICD13; Lane 5, ICD31; Lane 6, INED17; and Lane 7, INED33

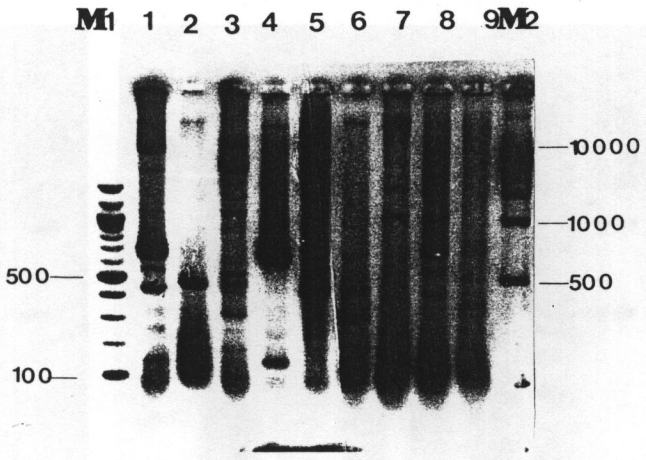

Figure 75. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, VND13; Lane 2, VND26; Lane 3, VND31; Lane 4, VCD11; Lane 5, VCD21; Lane 6, VCD31; Lane 7, VNED11; Lane 8, VNED22 and Lane 9, VNED37

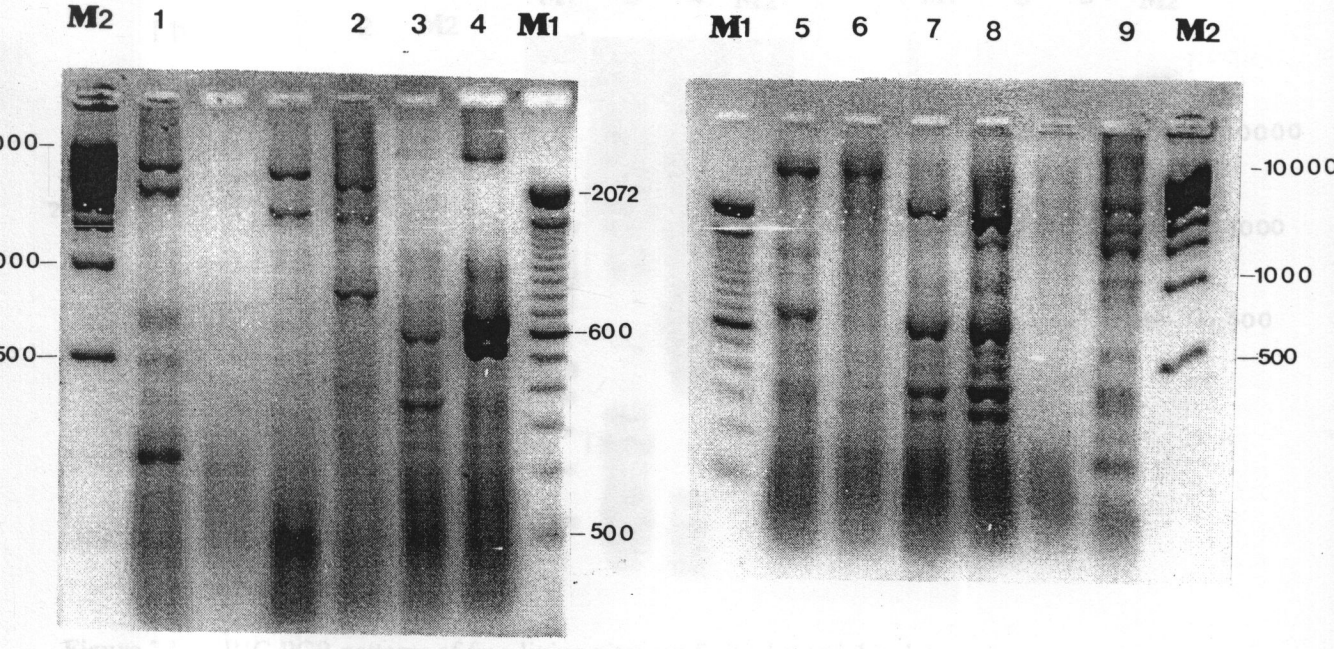

Figure 76. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, INA19; Lane 2, ICA23; Lane 3, INEA17; Lane 4, INEA27; Lane 5, VNA13; Lane 6, VNA23; Lane 7, VCA12; Lane 8, VCA22 and Lane 9, VNEA21

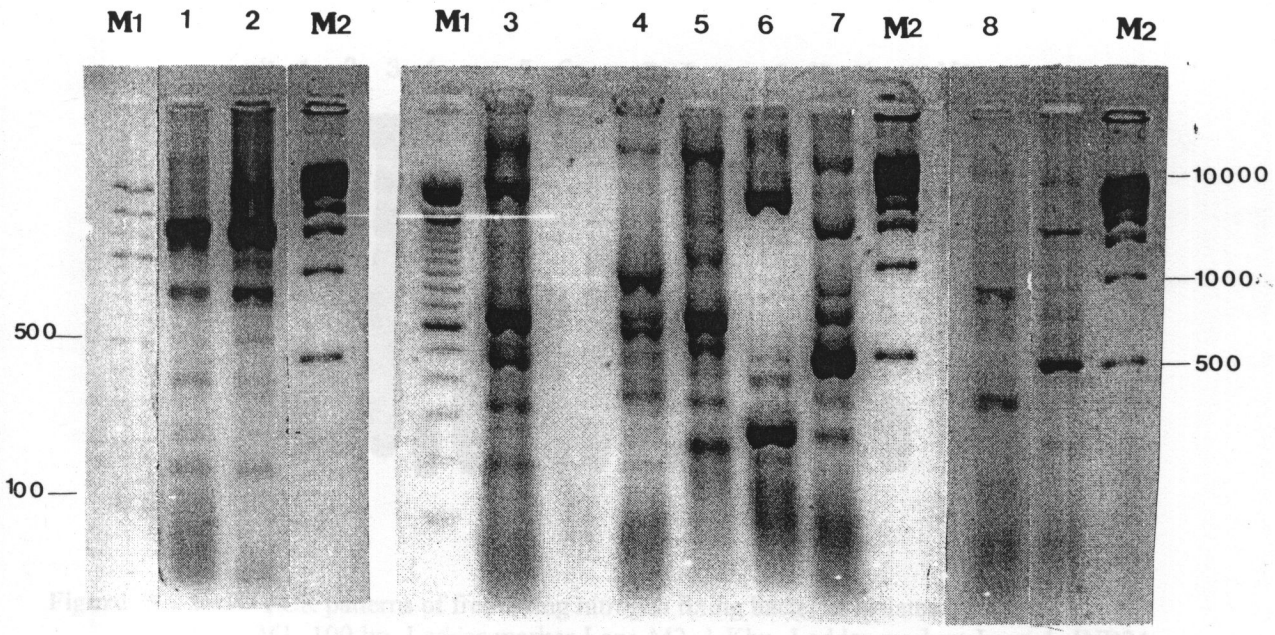

Figure 77. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, ICB12; Lane 2, ICB27; Lane 3, VNB14; Lane 4, VCB11; Lane 5, VCB23; Lane 6, VNEB14 Lane 7, VNEB21 and Lane 8, VNB21

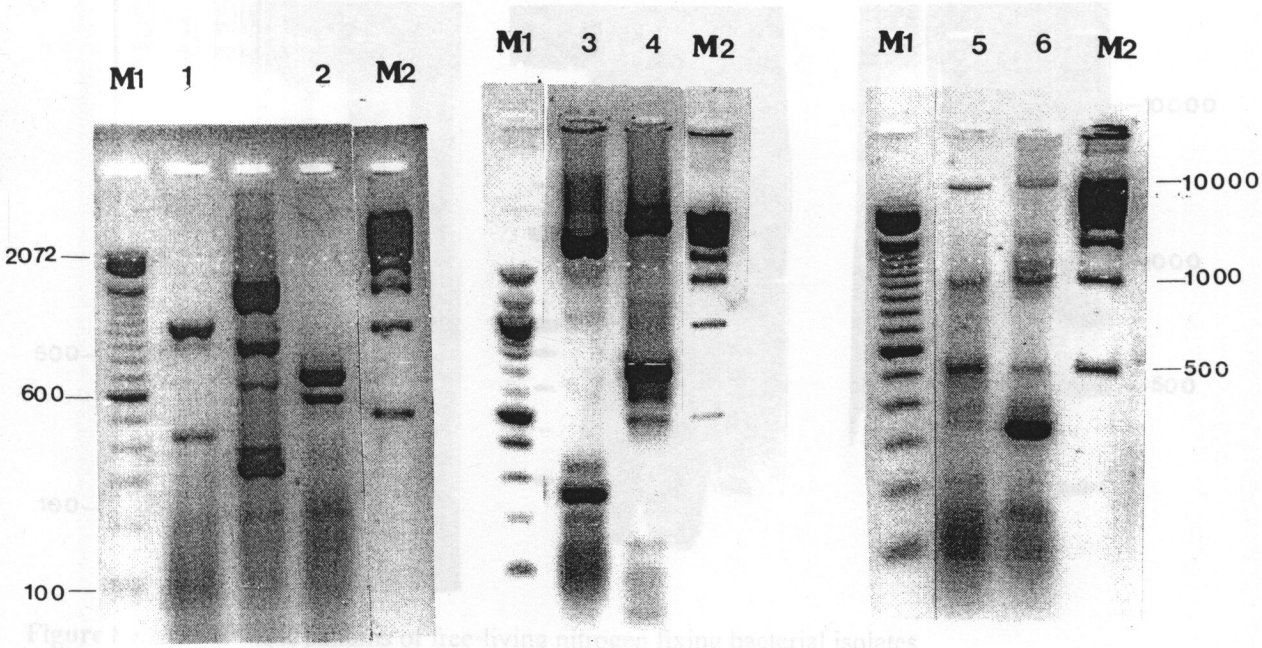

Figure 78. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, INC*16; Lane 2, ICC*13; Lane 3, VCC*11; Lane 4, VCC*22; Lane 5, VNEC*11 and Lane 6, VNEC*21

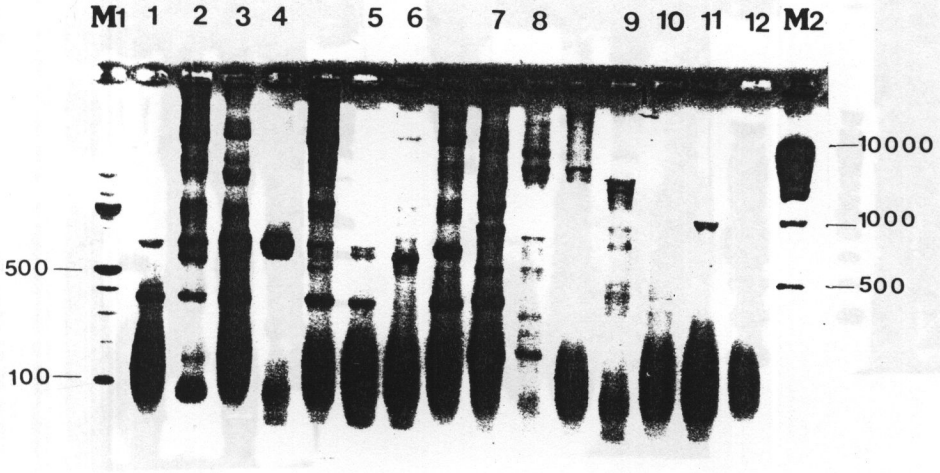

Figure 79. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, INB24; Lane 2, INEB13; Lane 3, INM213; Lane 4, IVNEM211; Lane 5, INECR37 Lane 6, INECR11; Lane 7, INEC*23; Lane 8, INEM236; Lane 9, IVNEM231; Lane 10, IVNCR22; Lane 11, IVNEC12 and Lane 12, IVNC31

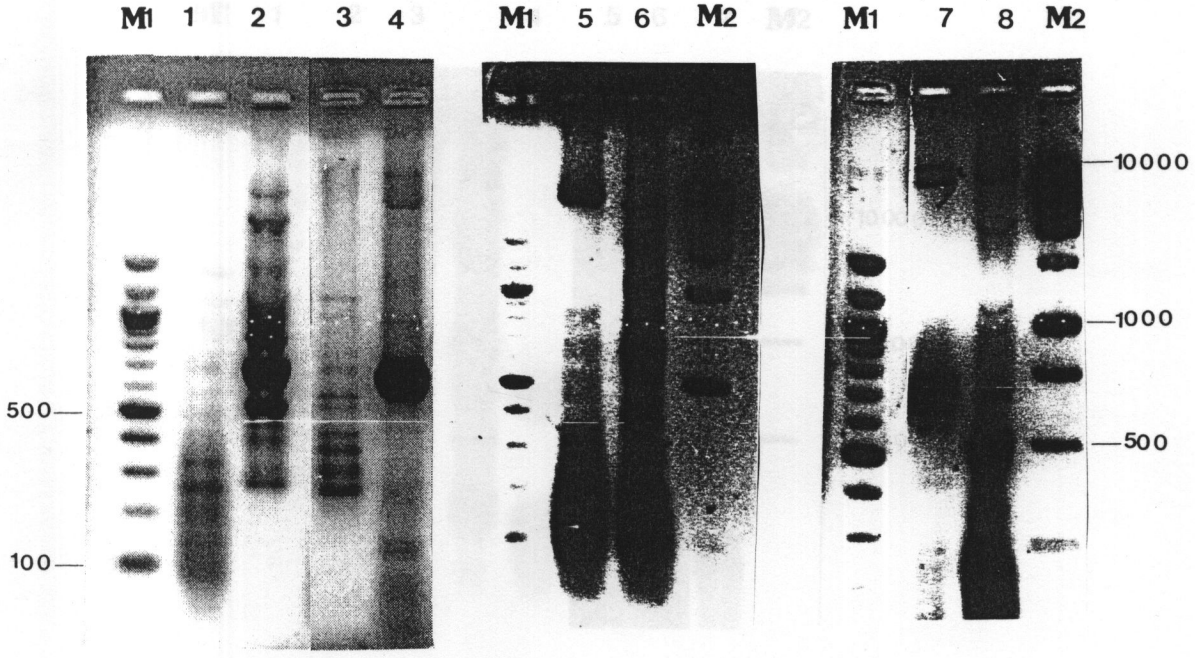

Figure 80. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, IVNR26; Lane 2, IVNEM224; Lane 3, ICM312; Lane 4, INEM216; Lane 5, INEC*16; Lane 6, ICC*25; Lane 7, IVCCR41 and Lane 8, IVCF42

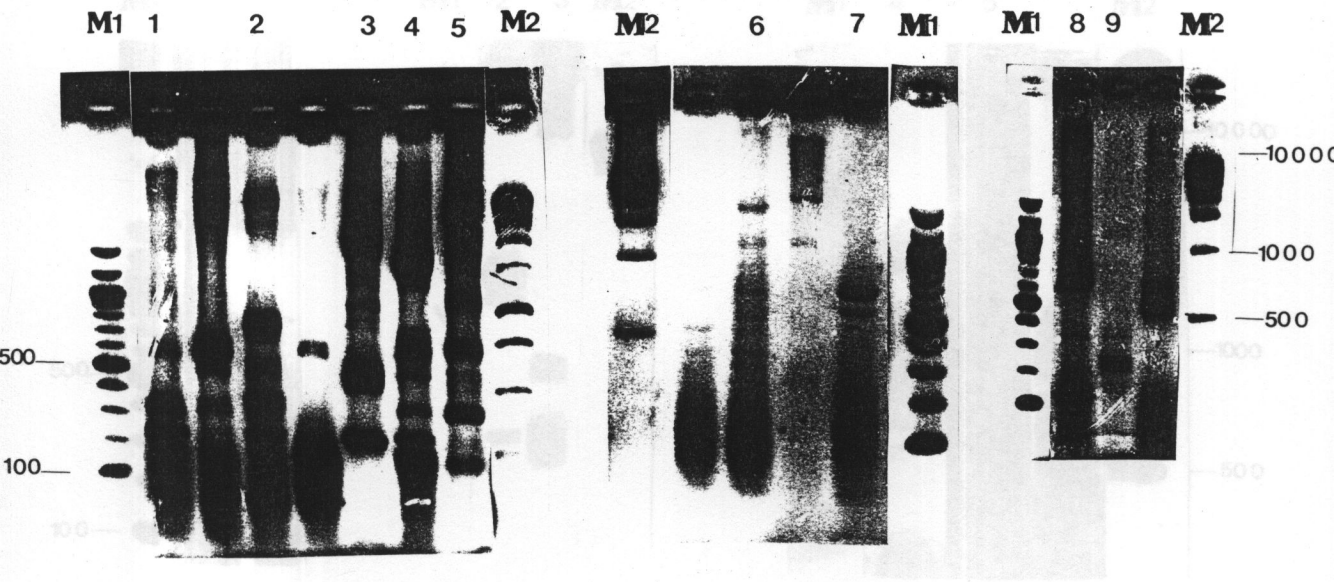

Figure 81. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, ICCR26; Lane 2, INECR22; Lane 3, IVNF11; Lane 4, INECR44; Lane 5, INER15; Lane 6, IVNC31; Lane 7, ICR35; Lane 8, VNC*24 and Lane 9, INEF27

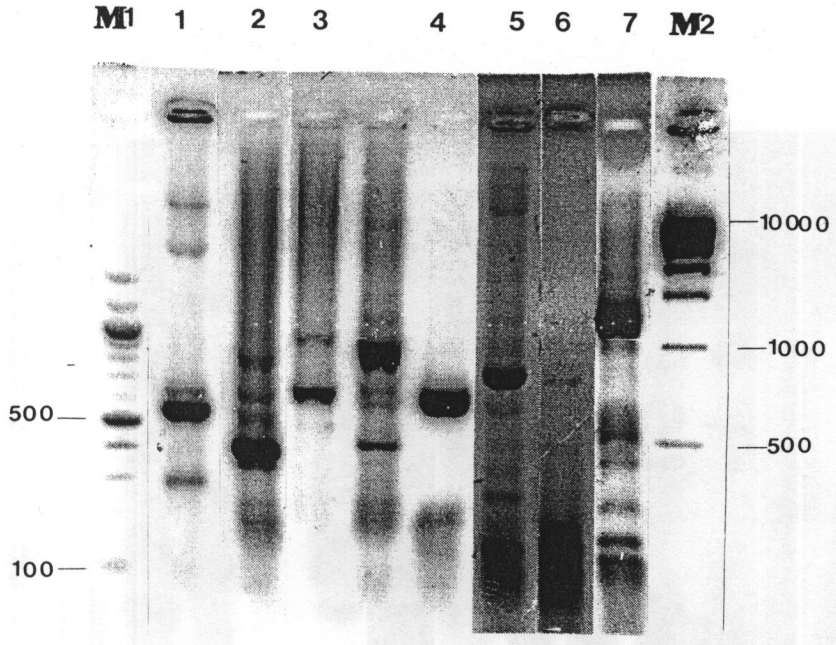

Figure 82. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, VNC*12; Lane 2, IVNCR11; Lane 3, IVCC24; Lane 4, INM314; Lane 5, IVNF24; Lane 6, INF47 and Lane 7, INEB11

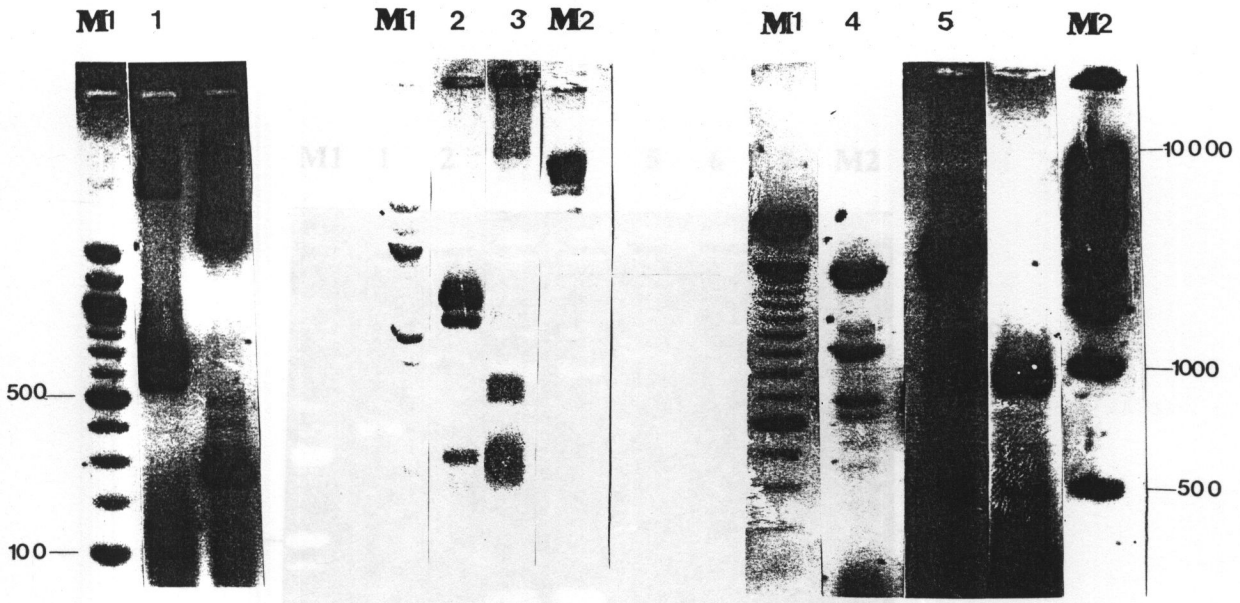

Figure 83. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, INEC48; Lane 2, INM343; Lane 3, ICM332; Lane 4, VNEA11 and Lane 5, INC*281

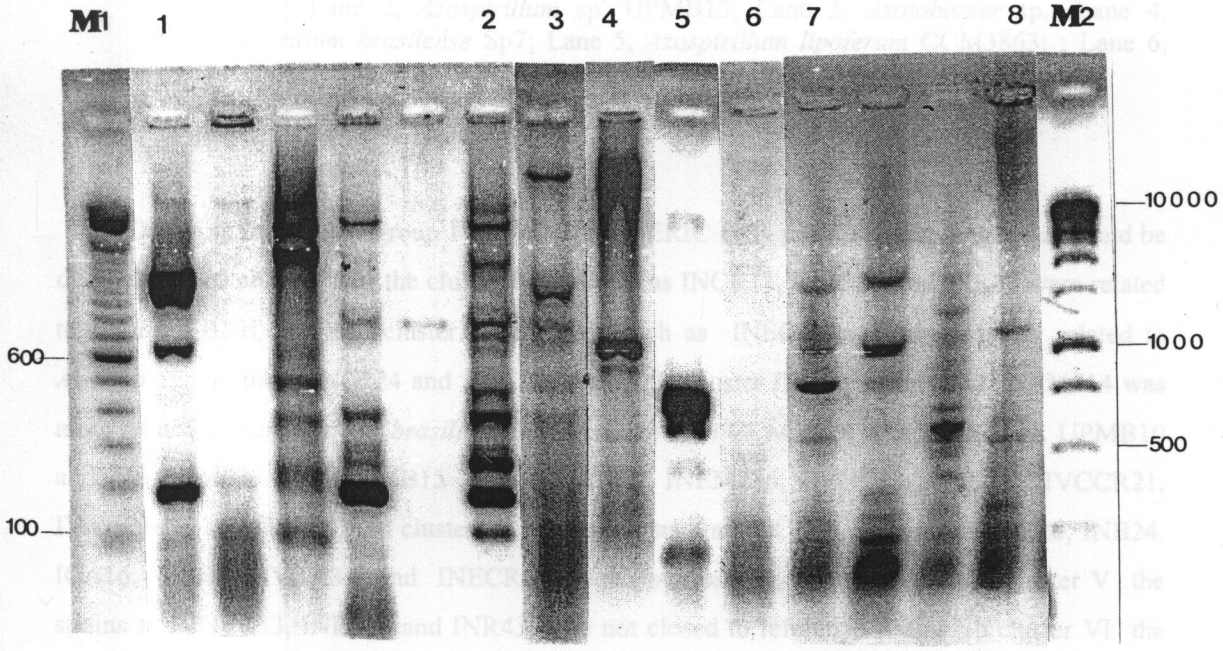

Figure 84. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, IVNC23; Lane 2, IVCC32; Lane 3, IVNEC22; Lane 4, IVCM233; Lane 5, ICD21; Lane 6, INC31; Lane 7, ICC34 and Lane 8, INEC23

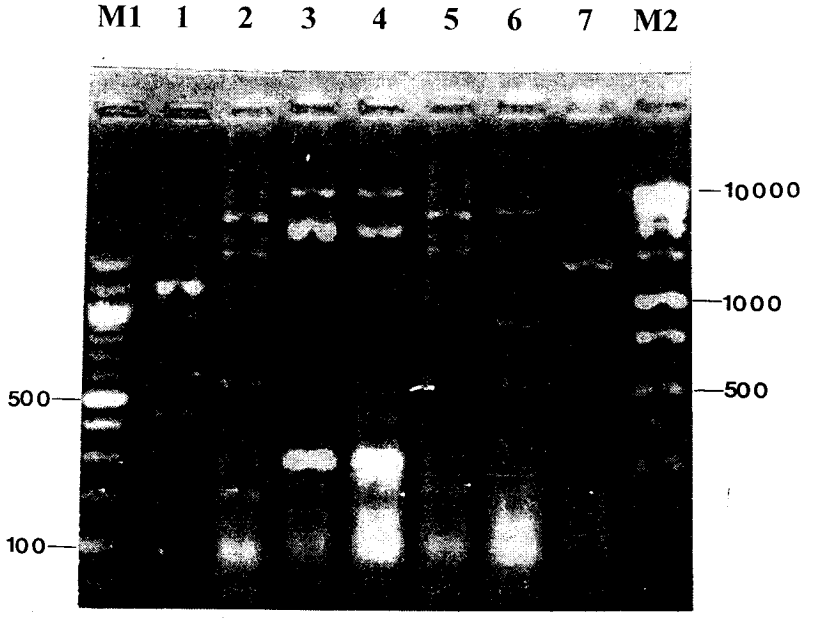

Figure 85. ERIC-PCR patterns of free-living nitrogen fixing bacterial isolates
Lane M1, 100 bp. Ladder marker Lane M2, 1 Kbp. Ladder marker; Lane 1, *E. coli* HB101; Lane 2, *Azospirillum* sp. UPMB13; Lane 3, *Azotobacter* sp.; Lane 4, *Azospirillum brasilense* Sp7; Lane 5, *Azospirillum lipoferum* CCM3863; ; Lane 6, *Azospirillum* sp. UPMB10 and Lane 7, *Beijerinckia* sp.

Dendrogram of *nif D* group 1 generated from ERIC-PCR products were found that could be divided into 10 clusters. For the cluster I the strains as INCR11, ICCR38 and INR33 were related to *E. coli* HB101 while in cluster II the strain such as INEC14 was more closely related to *Azotobacter* sp. than VNC*24 and INM116 strains. In cluster III the strains as IVNEM314 was more related to *Azospirillum brasilense* Sp7, A. *lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 than ICM231, INEM236, IVNC13, IND13, IVCCR21, IVNECR12, and IVNC31. In cluster IV the strains such as INCR24, INM111, ICCR26, INB24, ICA16, IVCR21, IVCR33 and INECR22 were not closed to reference strains. Cluster V the strains as IVNEC33, INEF47 and INR43 were not closed to reference strains. In cluster VI the strains such as INECR44, INEB 11, INM125, ICD21, ICB12, IVCF24, INEA27, ICD31, IND24 and ICB27 were not closed to reference strains. In cluster VII the strains as IVNEM133 and INF47 were not related to reference strains. Cluster VIII the strains as IVNEM341 and IVNR44 were not related to reference strains. In cluster IX the strain such as INEA17 was related to *Beijerinckia* sp.. Bacterial strains as IVNEM333, INC*26 and IVNR32 were belong in an another different group (Fig 88).

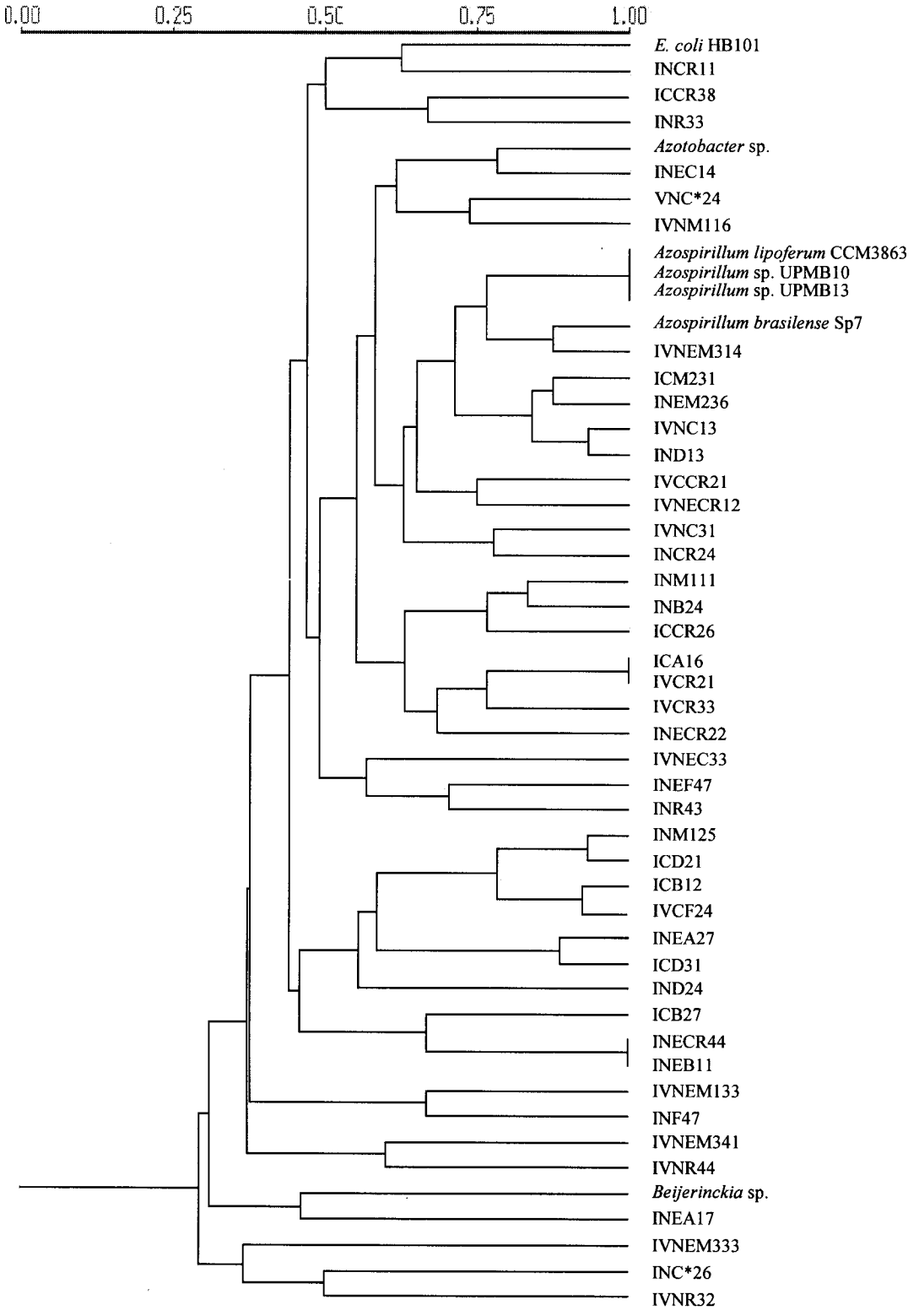

Figure 86. Dendrogram of group 1 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 2 was displayed that stains as INM145 and ICM249 were more related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 than other reference strains. (Fig. 87).

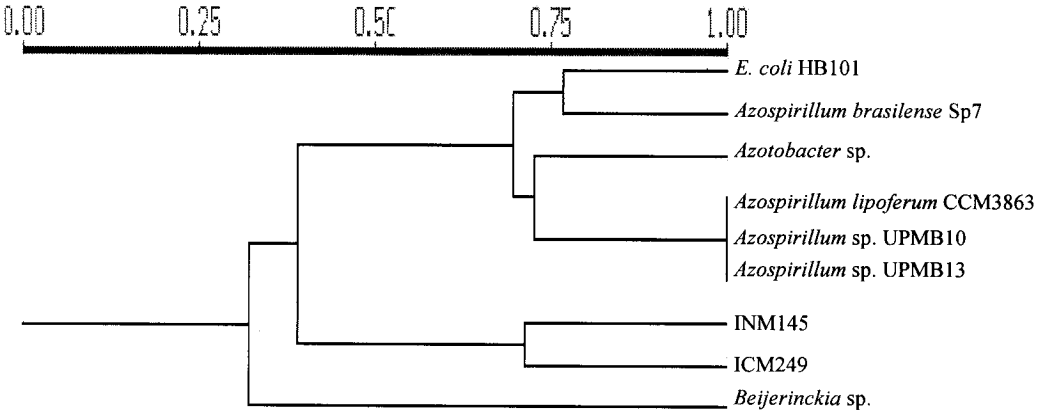

Figure 87. Dendrogram of group 2 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 3 was shown that bacterial isolate as ICM123 was distincted from *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13, *Beijerinckia sp.* and *Azotobacter* sp. while INED23 and IVNECR33 strains were more related to *Azotobacter* sp. than other (Fig. 88).

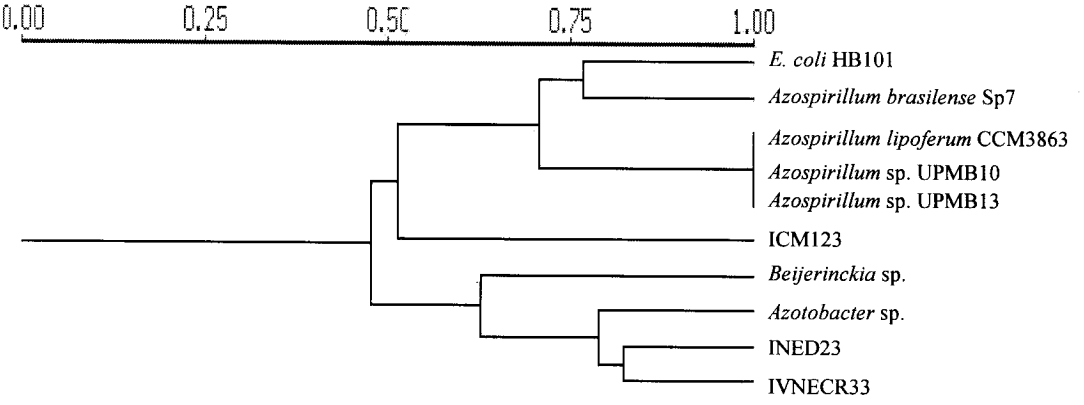

Figure 88. Dendrogram of group 3 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 4 the strains as INM133, ICM147 and VNED22 strains were distincted from *E. coli* HB101 and *Azospirillum brasilenese* Sp7 but more related with *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 than *Beijerinckia* sp. and *Azotobacter* sp. While strains ICC11, INED33, ICM321 and VCD11strains were more related to *Azotobacter* sp. than other. Strain IVNEM224 and IVNF41 were related to *Beijerinckia* sp.. For VCD21 and IVNEM112 strains were belong in an other different clades (Fig. 89).

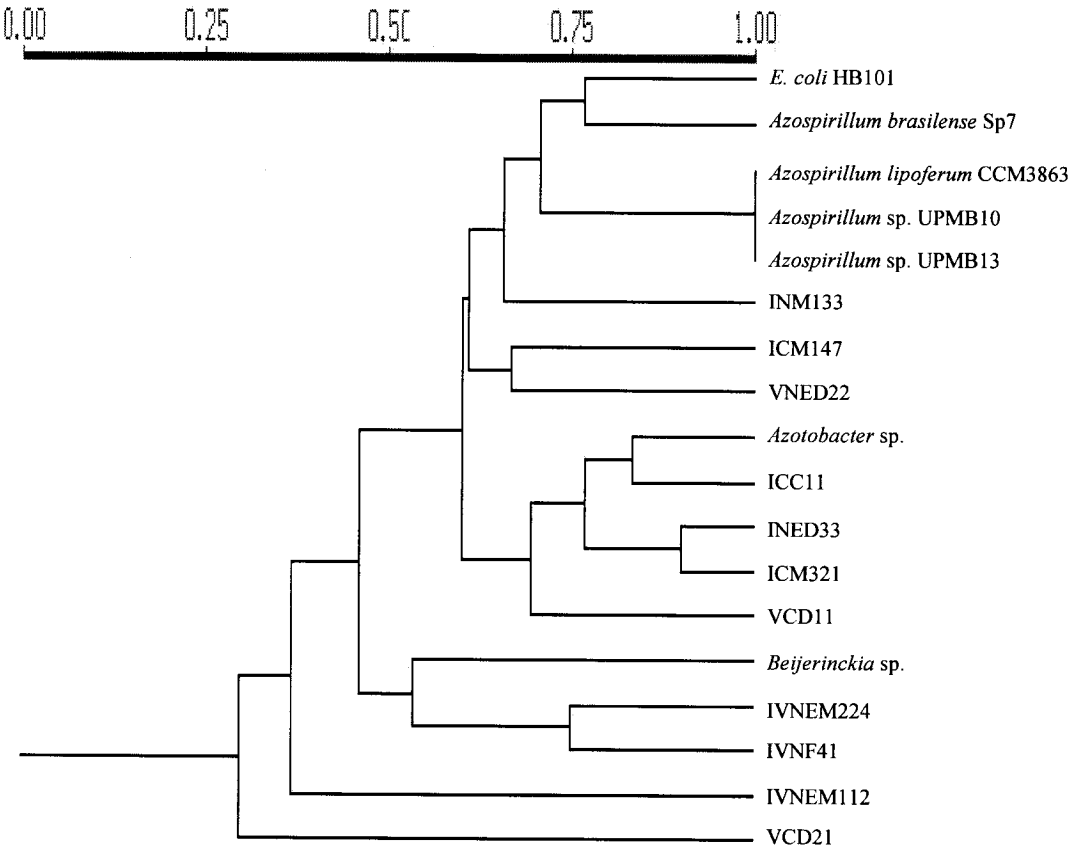

Figure 89. Dendrogram of group 4 separated *nif*D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 5 was displayed that stain as VNED37 were more related to *Azotobacter* sp. , *Azospirillum brasilenese* Sp7, *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 than VNEA11 and ICM118 and VCA12 strains but different from *Beijerinckia* sp. (Fig. 90).

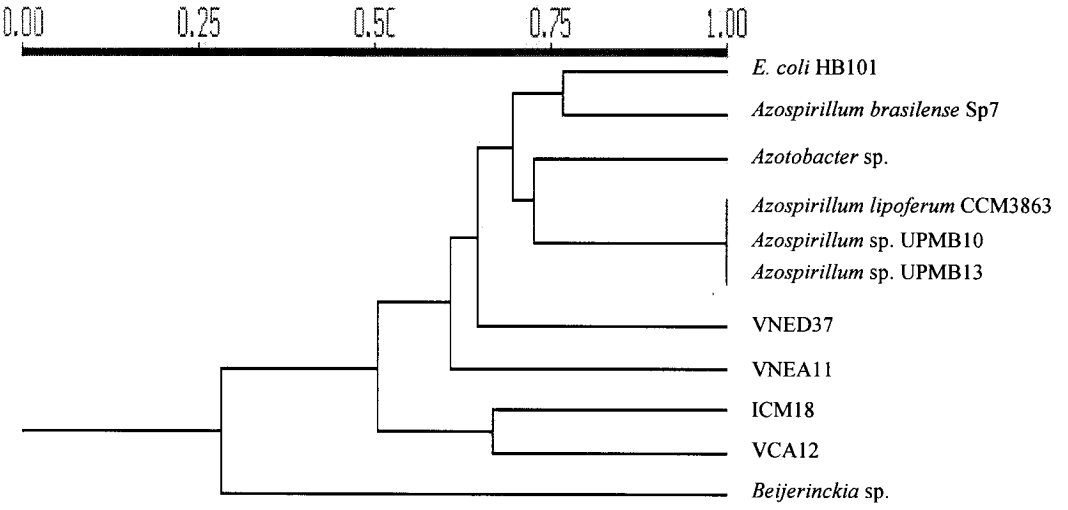

Figure 90. Dendrogram of group 5 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 6 was separated 3 clusters that cluster I the strains could be separated 3 groups by group 1.1 the strains as ICC34 was more related to *Azotobacter* sp. than ICF39, IND39 and ICF24 strains. Group 1.2 the strain as INEF27 was more related *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 than ICR12 and IVCR41 strains. And group 1.3 the strains such as ICR24, ICR35, IVNEC22, IVNEF24, ICM312, IVNECR45, INEM114, ICCR48, INER15, IVNEC12, ICF15, IVNC24, IVCF32, VNA13 IVNR26, INC31, IVNCR15, IVNEC43, INER24, INEB21, ICM332, IVNF31, INF25, INCR36, INEM323, ICM347, IVNF31 and INECE37. ICM137 and INED17 strains were not related to reference strains. Cluster II the strains as INA19 was more related to *Beijerinckia* sp. than VNEB21, IVNCR43, VNEC*21, IVCCR41 and IVNER42 strains. Cluster III the bacterial strain as INB13 was not related to reference strains. Cluster IV ICD13 bacterial strain was belong in an other different clades (Fig 91).

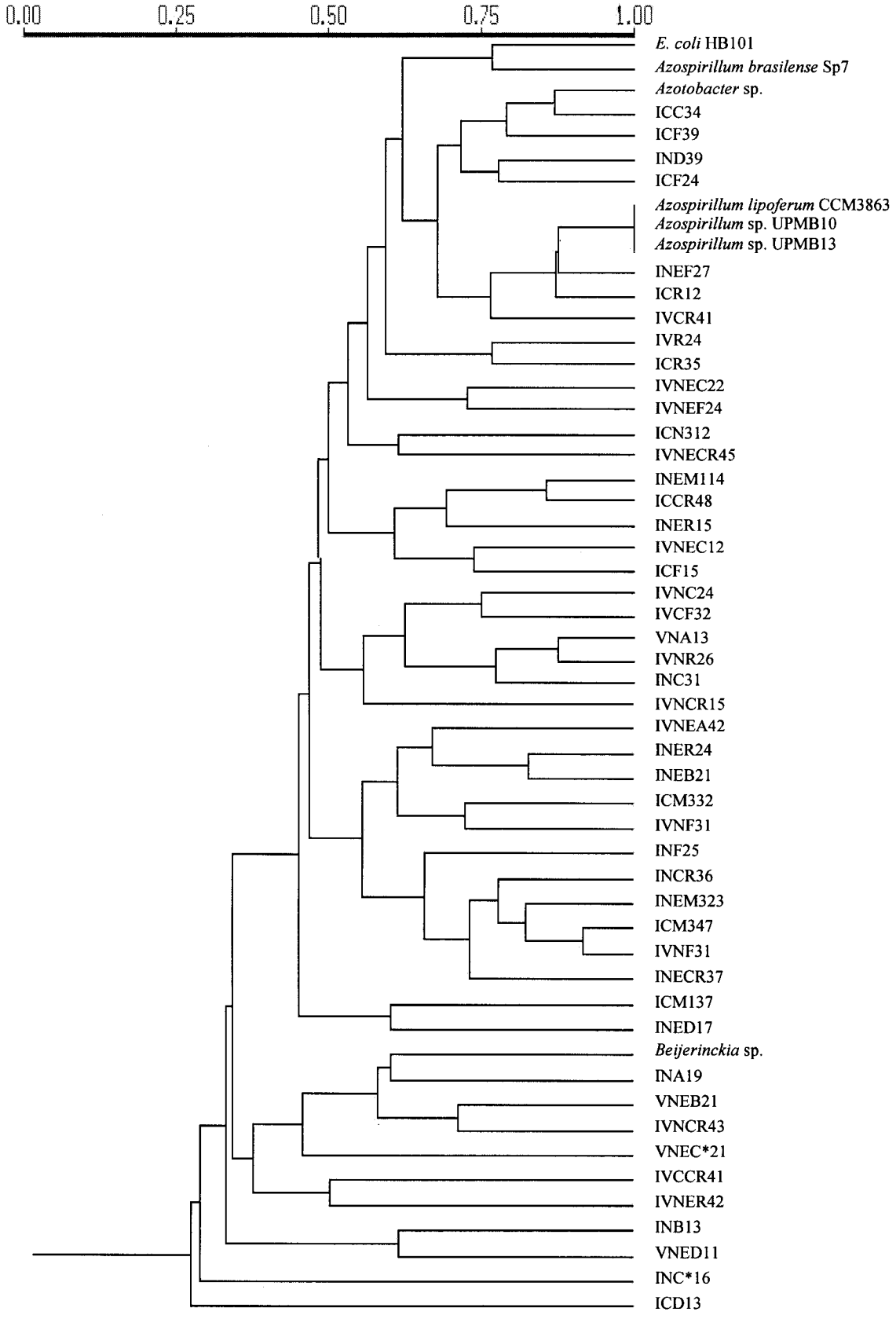

Figure 91. Dendrogram of group 6 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 7 was displayed that stain as INM248 was more related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13 and *Azotobacter sp.* than strain as IVNM331. And both of them were not related to *Beijerinckia* sp . (Fig. 92).

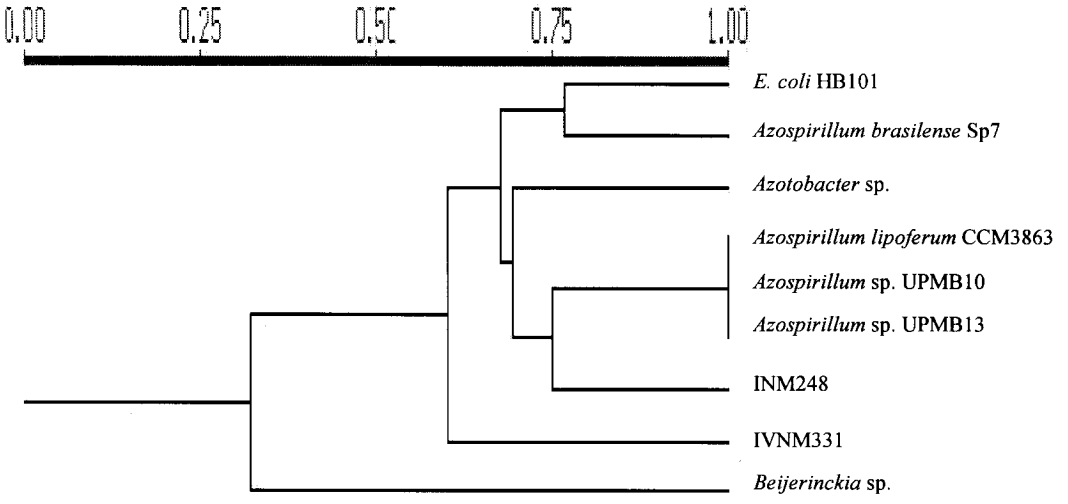

Figure 92. Dendrogram of group 7 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 8 was found that bacterial strain as INEM314 was related to *Azospirillum brasilense* Sp7. For strains VCA22 and INEM133 were more related to *Azotobacter* sp. than other refference strains. While INA23 and INM213 bacterial strains were related to *Azospirillum lipoferum* CCM 3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp UPMB13. (Fig. 93).

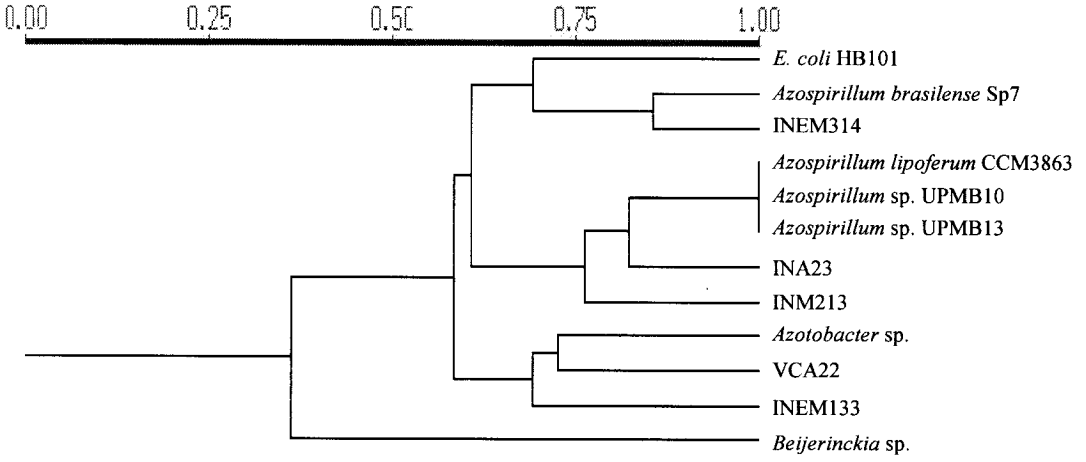

Figure 93. Dendrogram of group 8 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 9 was displayed that stains as IVNM212 was different to *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13 and *Azotobacter* sp.. While *Beijerinckia* sp. was out of clade (Fig. 94).

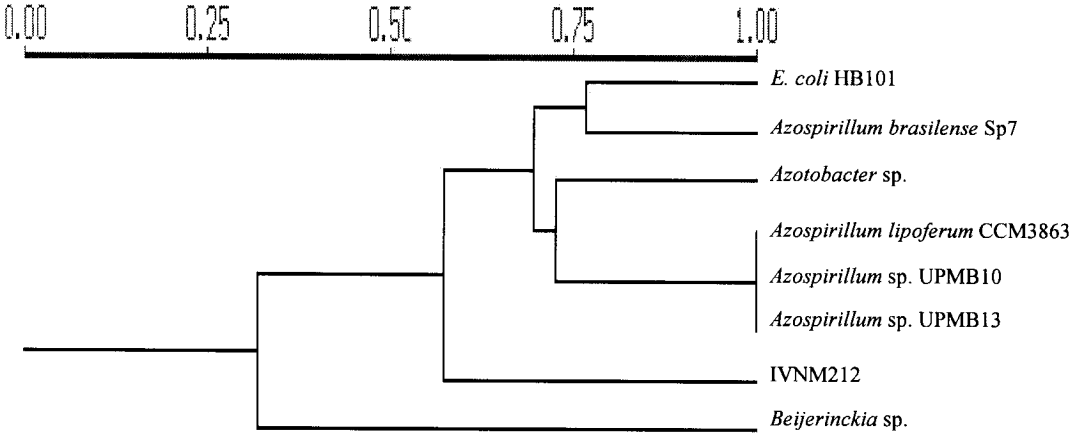

Figure 94. Dendrogram of group 9 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 10 was found that IVCM243 strain was more closed to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 than other reference strains (Fig. 95).

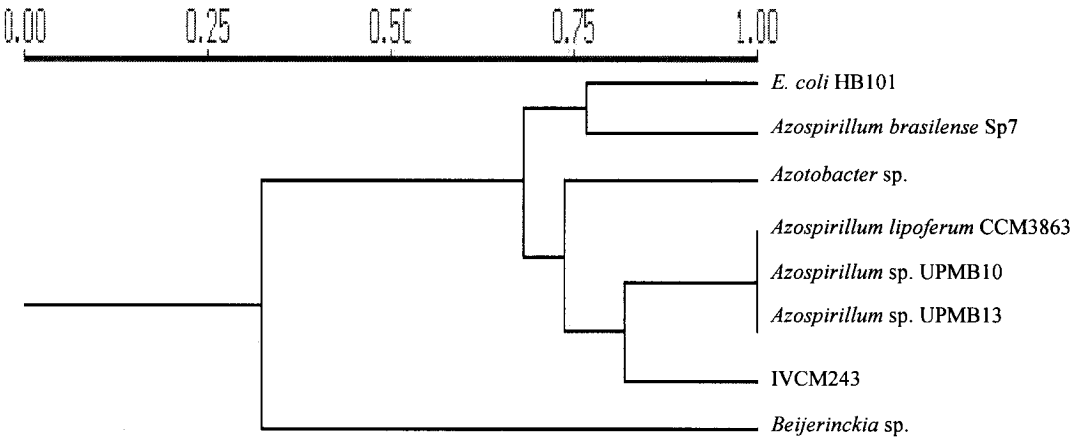

Figure 95. Dendrogram of group 10 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 11 was found that strain as IVNEM241 was different from *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13, *Beijerinckia* sp. and *Azotobacter* sp. while IVNER31 strain was belong in an another different group (Fig. 96).

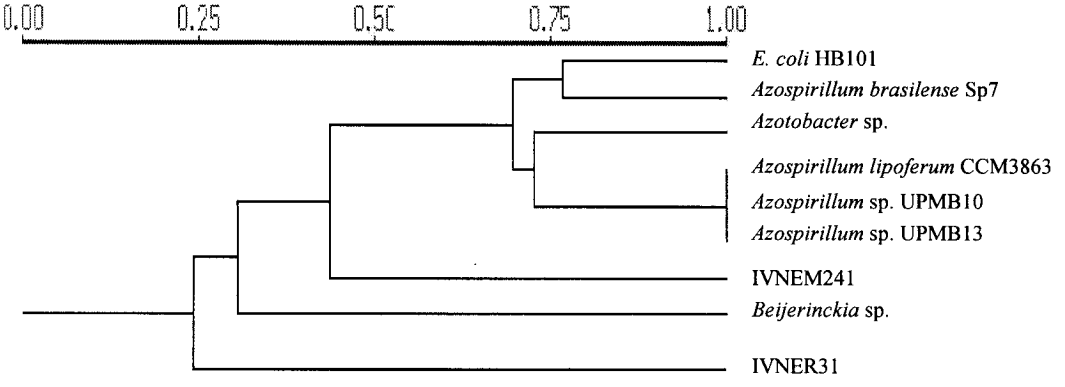

Figure 96. Dendrogram of group 11 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 12 was found that IVCF13 strain was belong in an another different group of reference strains (Fig. 97).

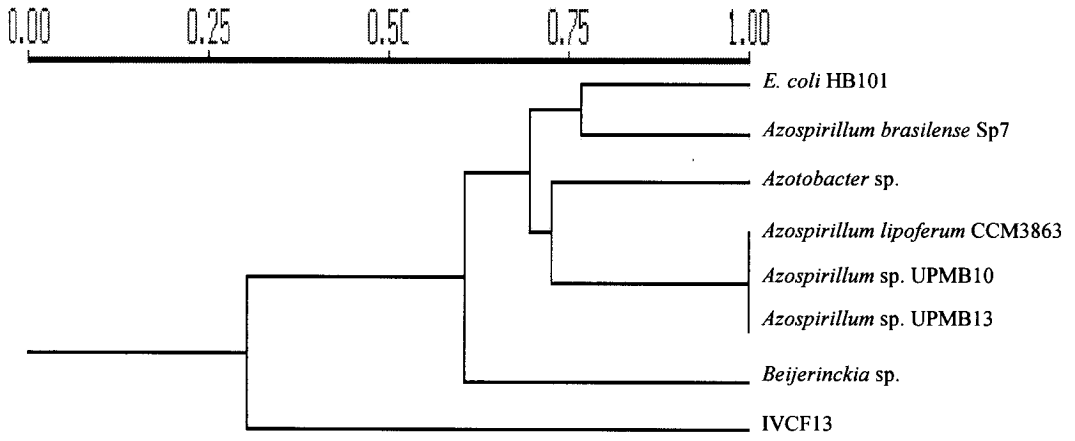

Figure 97. Dendrogram of group 12 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 13 was displayed that stains as IVCCR31 was more related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 than other reference strains (Fig. 98).

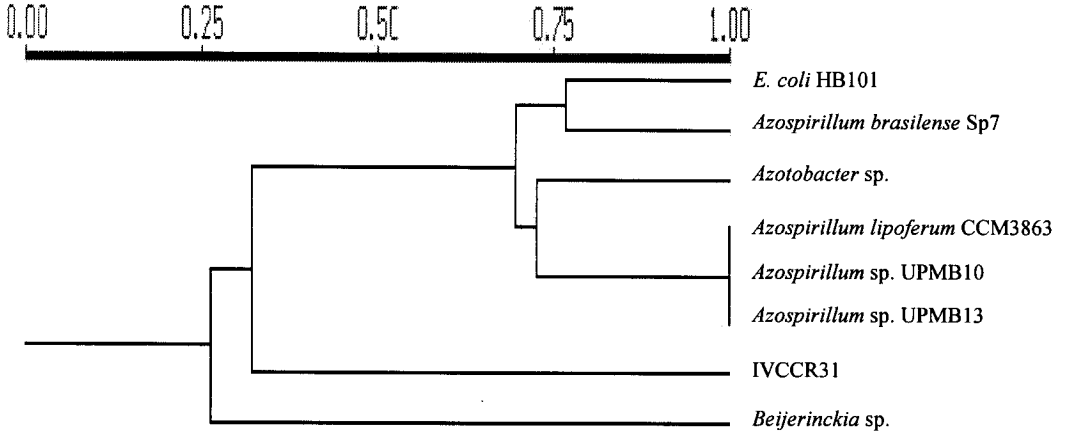

Figure 98. Dendrogram of group 13 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 14 displayed that stain as INEM123 was more related *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 than other reference strains (Fig. 99).

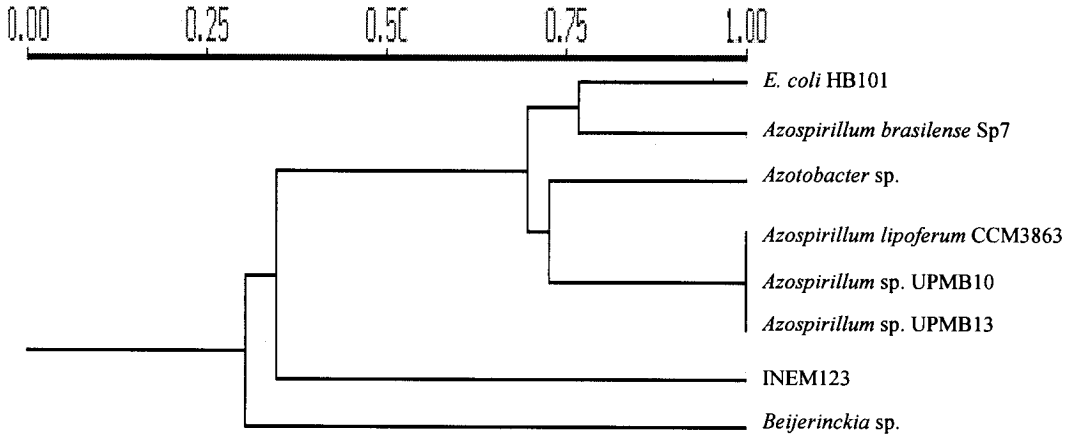

Figure 99. Dendrogram of group 14 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 15 was found that INEM143 strain was more closed to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 than other reference strains (Fig. 100).

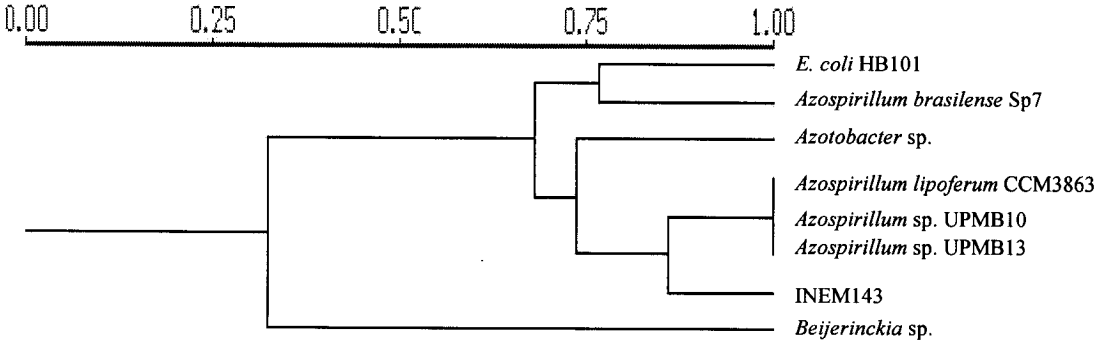

Figure 100. Dendrogram of group 15 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 16 found that could be divied into 6 clusters. The cluster I strain as IVNM224 strain was related to *E.coli* HB101 and *Azospirillum brasilense* Sp7. The strain in cluster II as IVCC24 was more closed to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 than INR25 and VCB23 strains. cluster III the strain such as ICF13 was related to reference strains and cluster IV the strains as INEM223, IVCC13 and IVCC32 were not related to reference strains . cluster IV the strains as IVNECR22 and INM224 were not related to reference strains. While cluster VI bacterial strain as ICM219 was related to *Beijerinckia* sp. (Fig. 101).

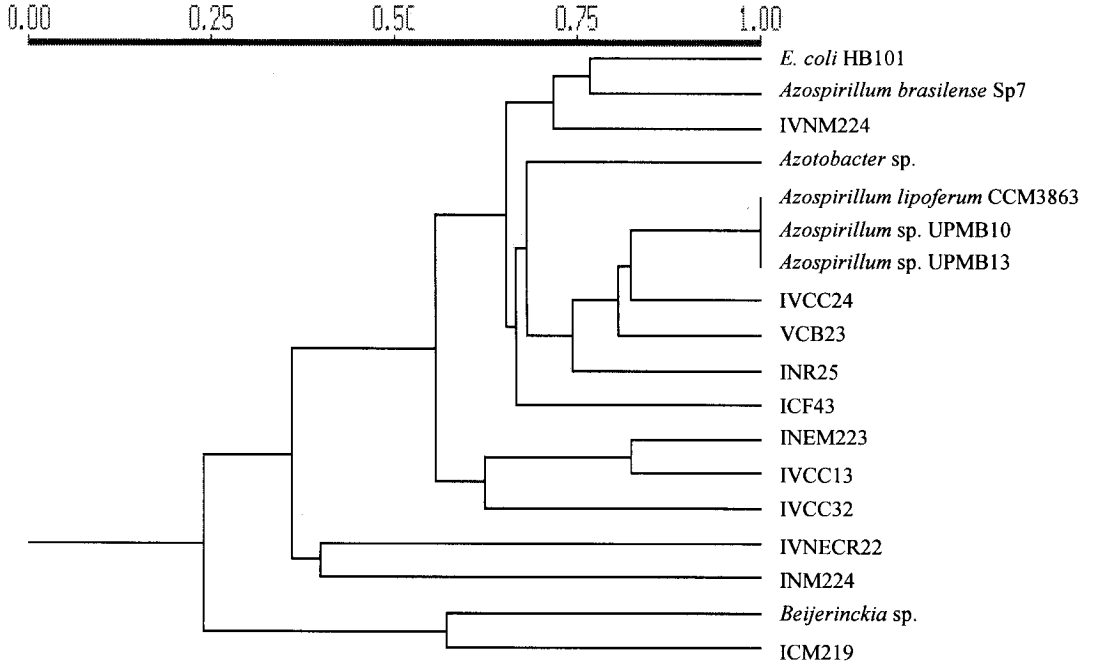

Figure 101. Dendrogram of group 16 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 17 was shown that isolated strain as INC15 was more related to *Azotobacter sp.* than other reference strains. While strain as INM236 was different to *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13, *Beijerinckia* sp. and *Azotobacter* sp. (Fig. 102).

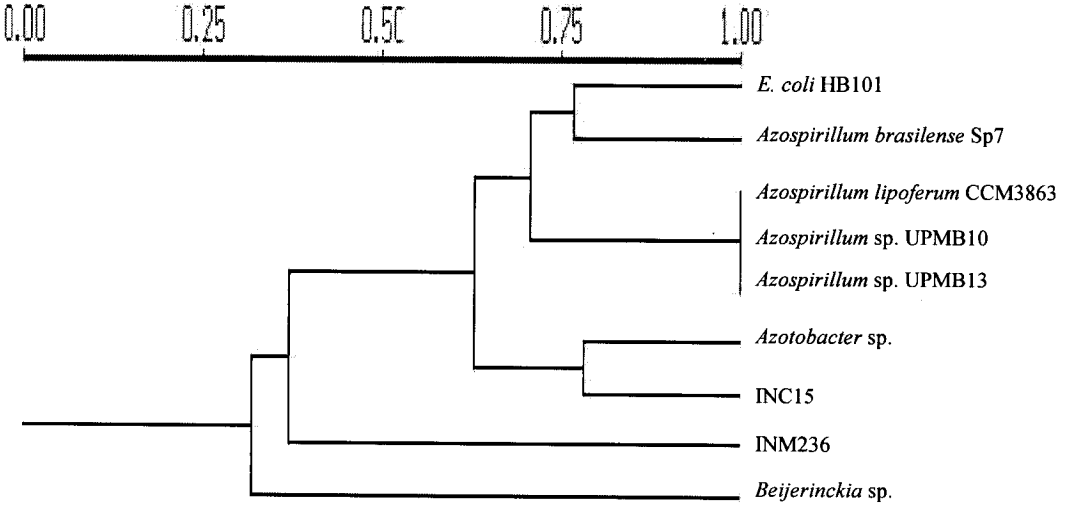

Figure 102. Dendrogram of group 17 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 18 was found that VCD31 strain was more related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 than other reference strains (Fig. 103).

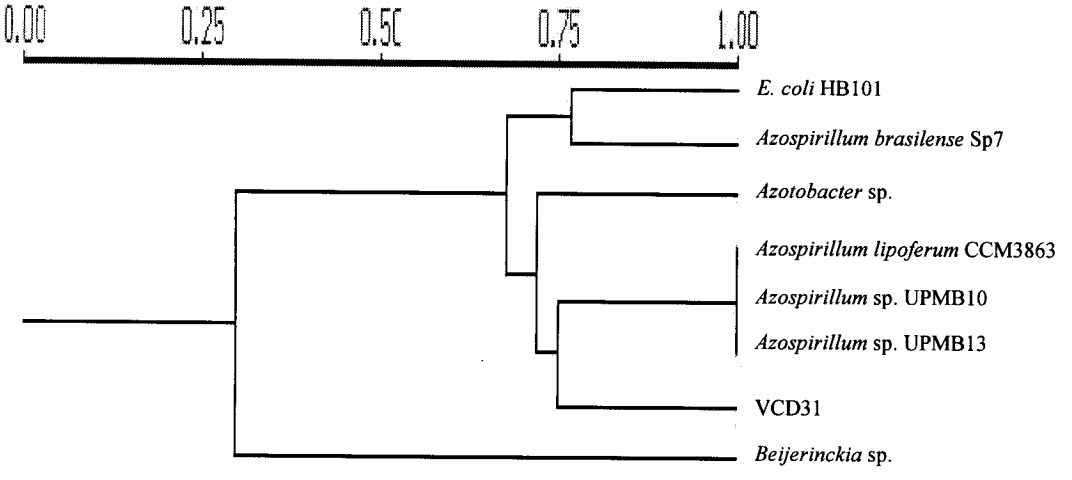

Figure 103. Dendrogram of group 18 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC prime

Dendrogram of group 19 was found that isolated strain as IVNCR22 was belong in an another different group (Fig. 104).

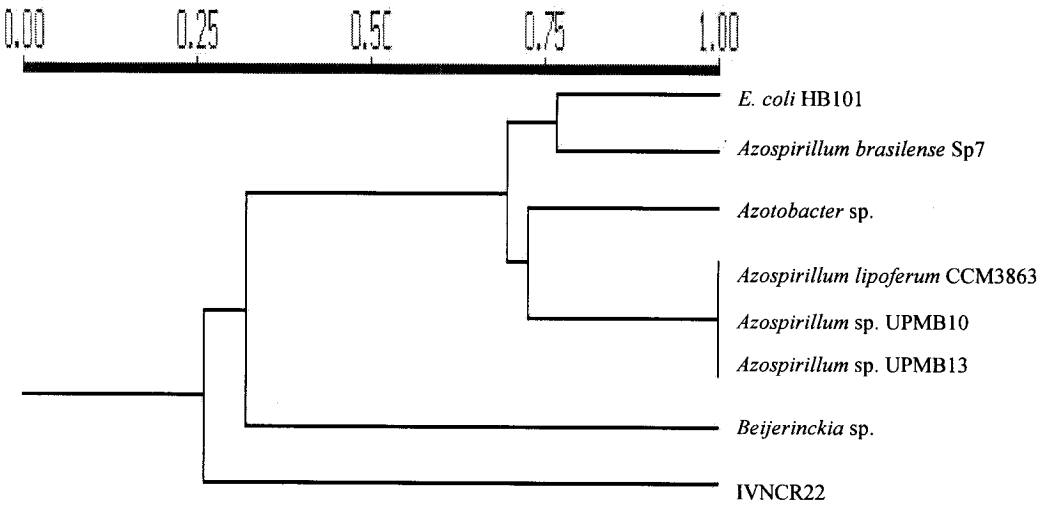

Figure 104. Dendrogram of group 19 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogramof group 20 was displayed that stain as IVNCR34 was more related to *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13. and *Azotobacter* sp. than *Beijerinckia* sp. (Fig. 105).

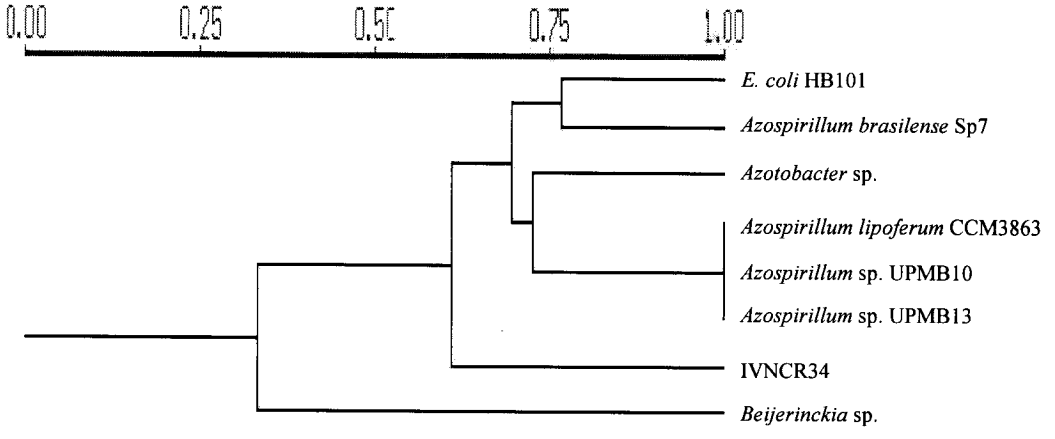

Figure 105. Dendrogram of group 20 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 21 found that IVCM 315 strain was closely related to *Azotobacter* sp. UPMB13 than other reference strains (Fig 106).

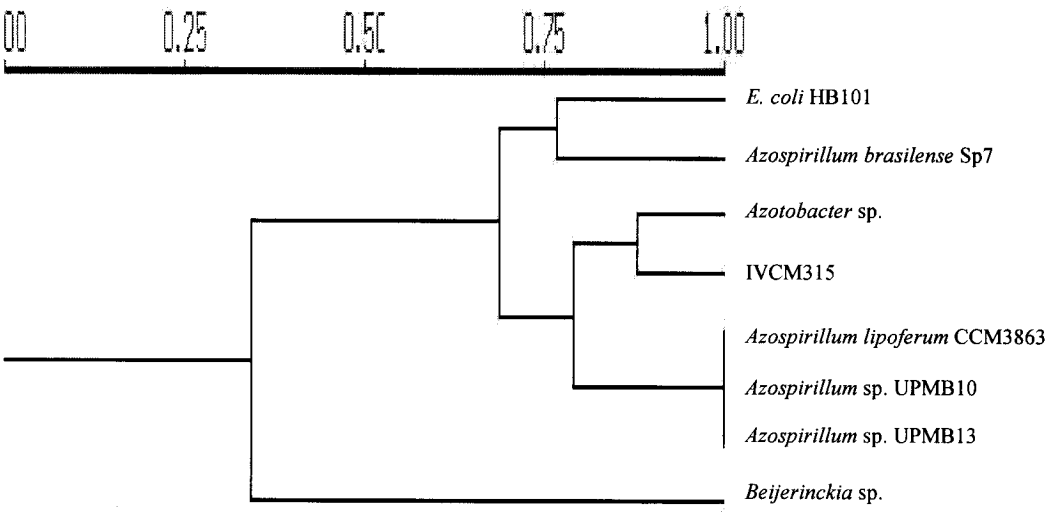

Figure 106. Dendrogram of group 20 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 22 found that could be separated 4 clusters. The The cluster I strain as ICCR16 strain was related to *E.coli* HB101 *Azospirillum brasilense* Sp7. Cluster II the strains such as IVCM233, IVCM323, IVNF24 and IVCM341 were more closely related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 than other reference strains. While clusterIII the strains as INEM244, IVNEM324 and INEM345 were not related to reference strains. The cluster IV the strains as VND26 was related to *Beijerinckia* sp. (Fig. 107).

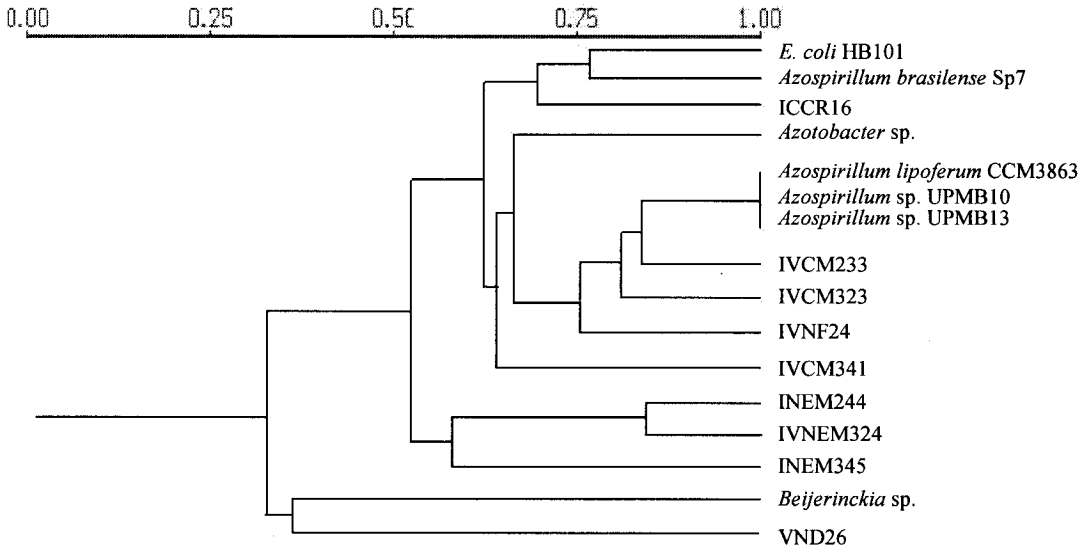

Figure 107. Dendrogram of group 22 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 23 was indicated that IVNC41 strain was related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 while IVNM311 and IVNM341 were different from *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13, *Beijerinckia* sp. and *Azotobacter* sp.. But IVNM322 strain was more related to *Beijerinckia* sp. than other reference strains (Fig. 108).

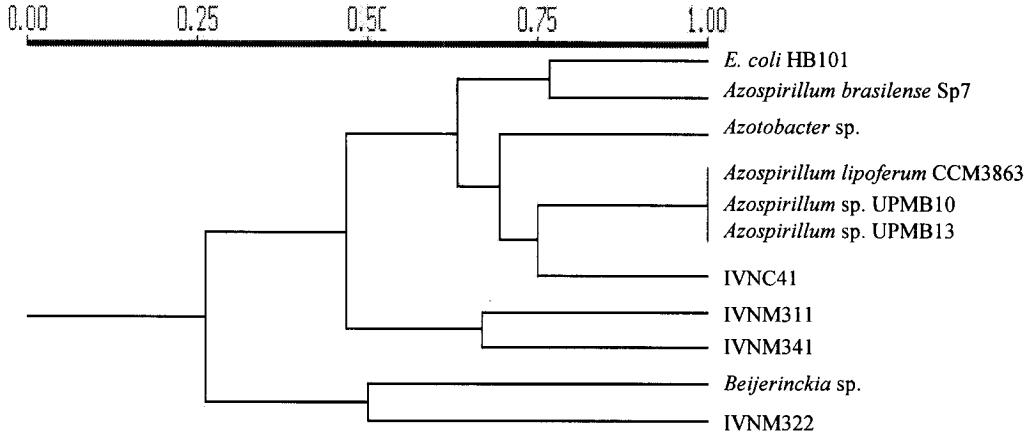

Figure 108. Dendrogram of group 23 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 24 was found that IVCC45 strain was related to INM314 strain and different from other reference strains except *Beijerinckia* sp. (Fig. 109).

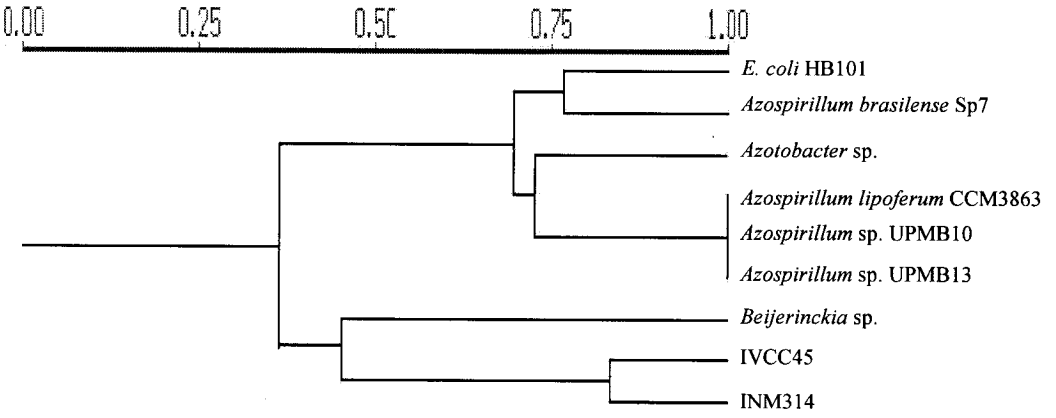

Figure 109. Dendrogram of group 24 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 25 was displayed that stains as VNC*12 and ICR44 were closely related to other reference strains except *Beijerinckia* sp.. The strains such as IVNEM211, IVNM243, INEF33 and IVNF11 were not related to other reference strains (Fig. 110).

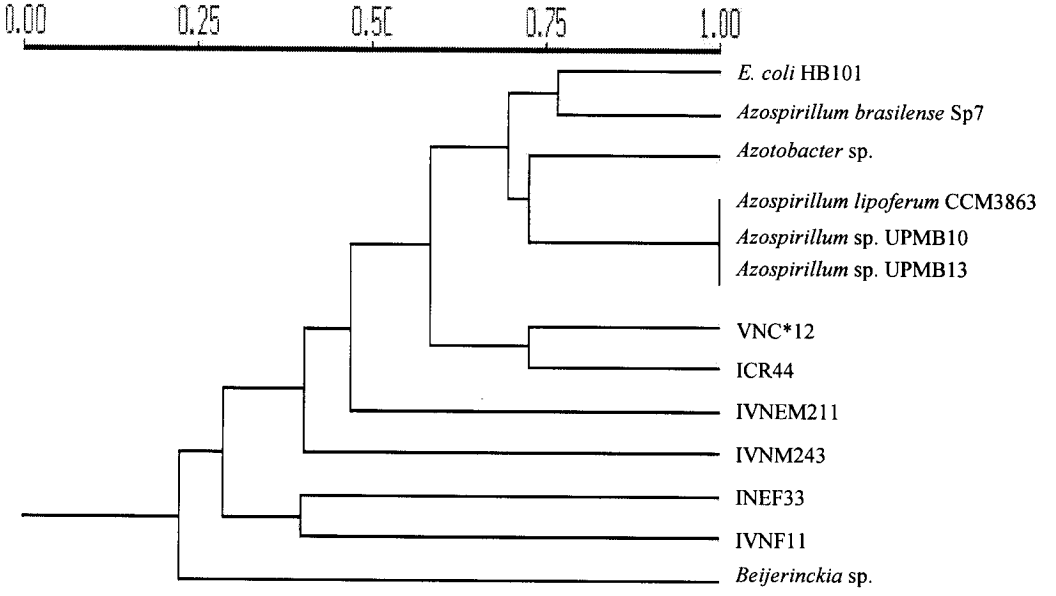

Figure 110. Dendrogram of group 25 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 26 was displayed that stain as INC29 was related to *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13and *Azotobacter* sp. except *Beijerinckia* sp. (Fig. 111).

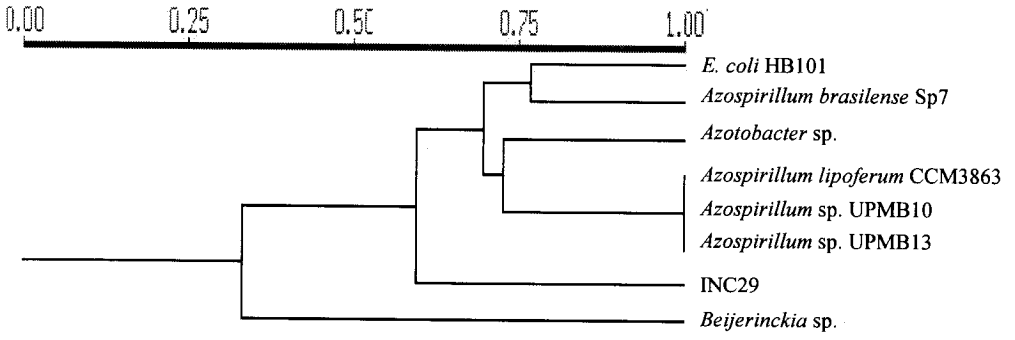

Figure 111. Dendrogram of group 26 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 27 was displayed that stain as IVCM213 was related to *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13and *Azotobacter* sp. except *Beijerinckia* sp. (Fig. 112).

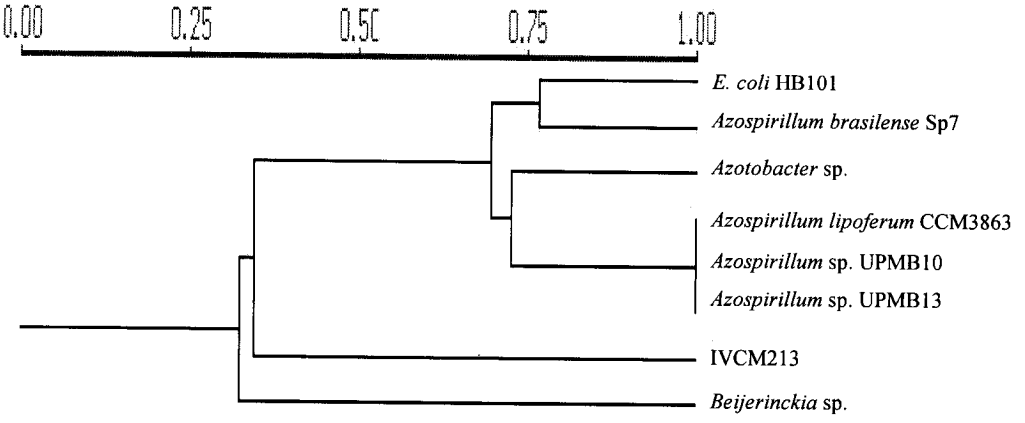

Figure 112. Dendrogram of group 27 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 28 was observed that bacterial strains as VNEB14 and IVNEF12 was more related to *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13 and *Azotobacter* sp. than INEC27 and INEC33. VCD11 strain was more related to *Beijerinckia* sp. than other reference strains (Fig. 113).

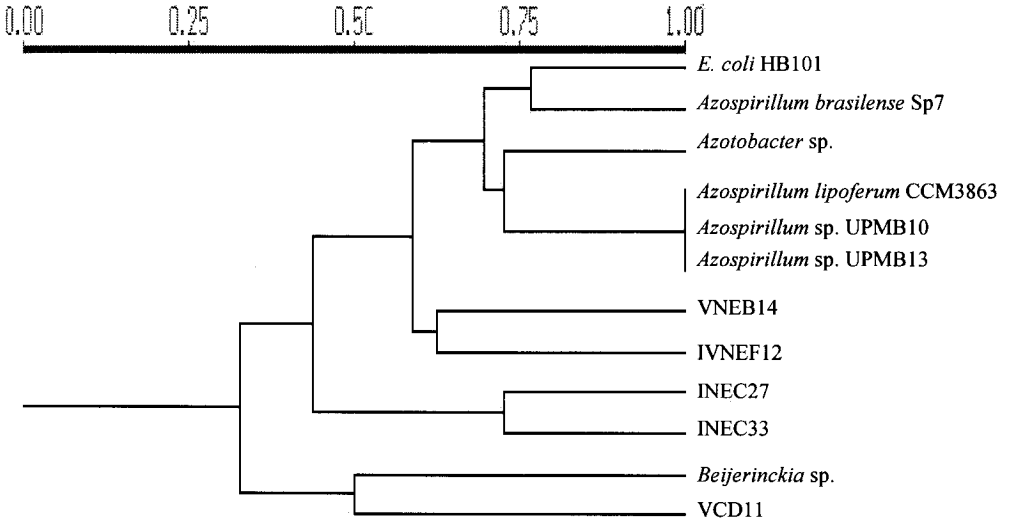

Figure 113. Dendrogram of group 28 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 29 was found that IVCF42 strain was more related to *Beijerinckia* sp. than other reference strains (Fig. 114).

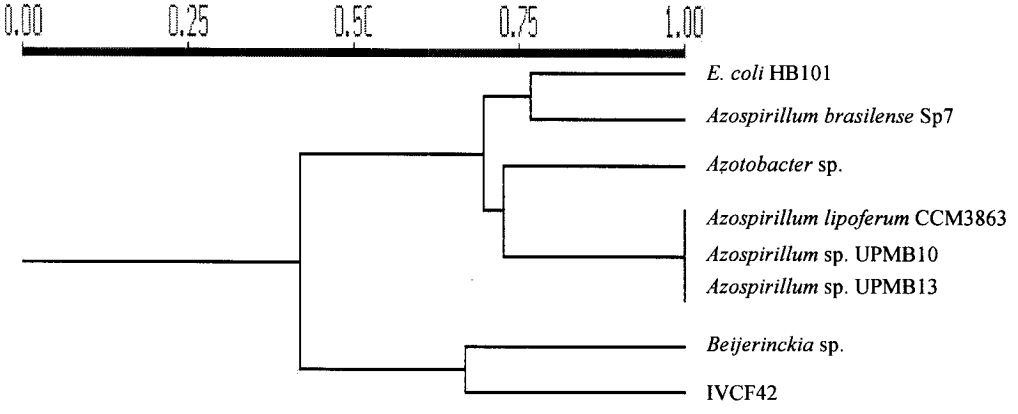

Figure 114. Dendrogram of group 29 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 30 was displayed that stain as IVCM335 was in the same clade as *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13 and *Azotobacter* sp. but not related to *Beijerinckia* sp. (Fig. 115).

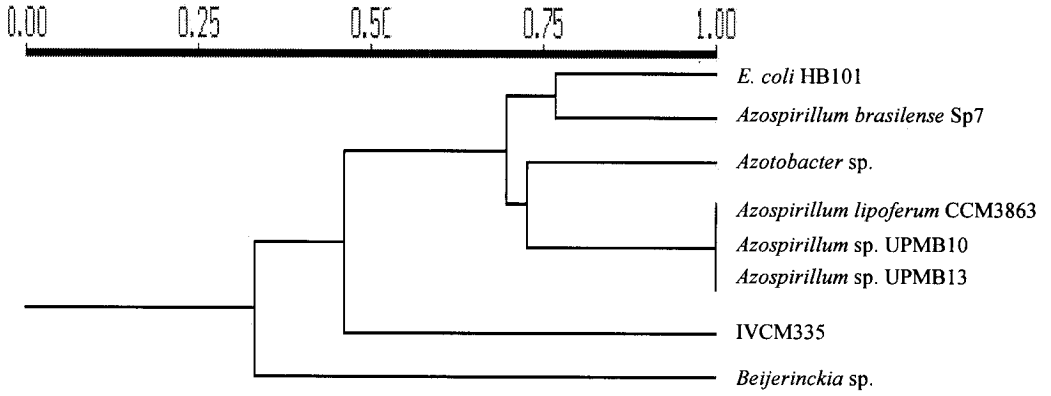

Figure 115. Dendrogram of group 30 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 31 was observed that VNB14 strain was more closely related to *Azotobacter* sp. than other reference strains. While bacterial strain as INCR410 and INER46 were neared to *Beijerinckia* sp. Bacterial strain ICC*13 as was belong in an other different clade (Fig. 116).

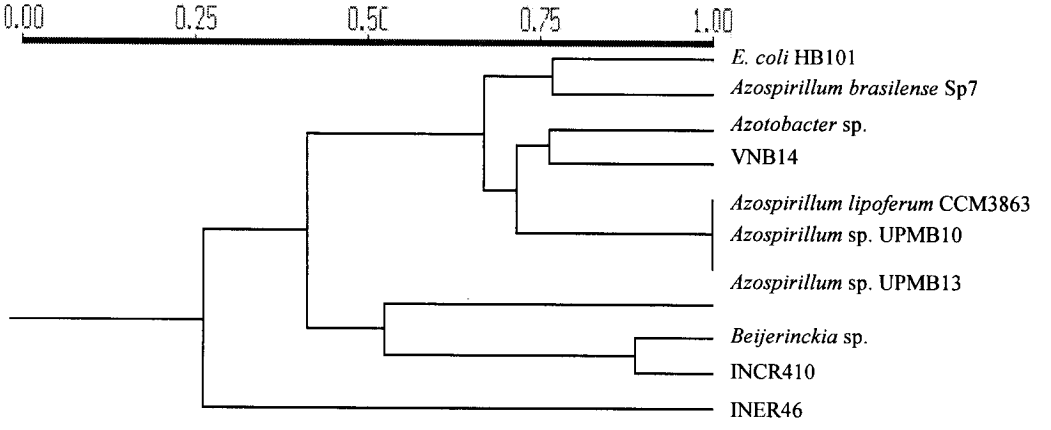

Figure 116. Dendrogram of group 31 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 32 was displayed that stain as IVNEF42 was in same group as *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13 and *Azotobacter* sp. but not related to *Beijerinckia* sp. (Fig. 117).

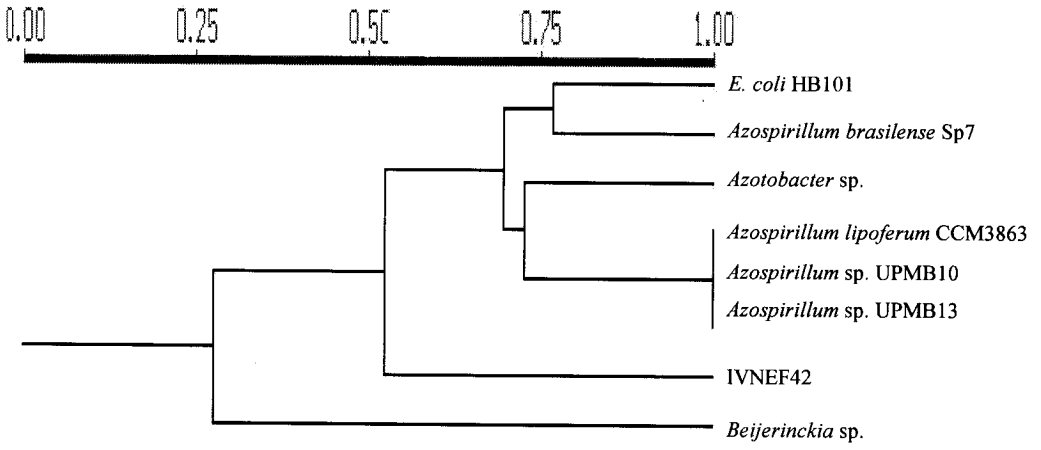

Figure 117. Dendrogram of group 32 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 33 found that strain as INEC*16 and ICM222 were more related to *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13, *Beijerinckia* sp. and *Azotobacter* sp. than VNB21 strain. INEC47 bacterial strain was related to *Beijerinckia* sp.(Fig. 118).

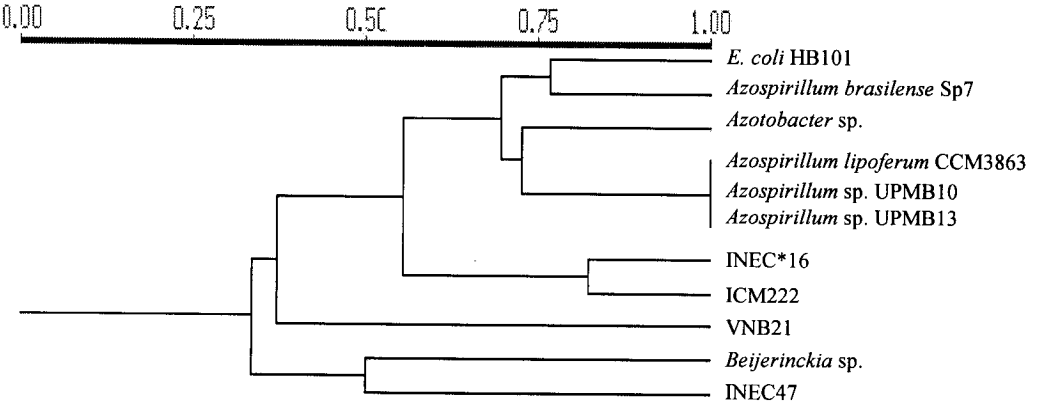

Figure 118. Dendrogram of group 33 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 34 was observed that IVCM332 strain was more related to *Beijerinkhia* sp. than other reference strains (Fig. 119).

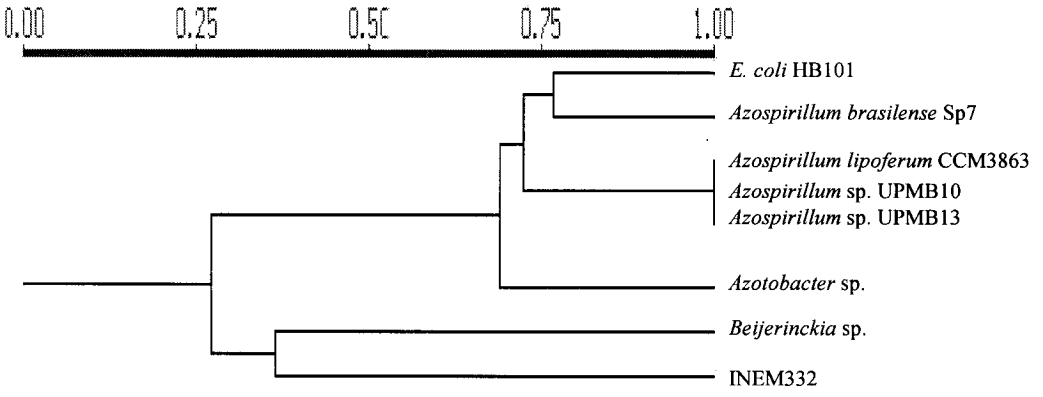

Figure 119. Dendrogram of group 34 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 35 was found that bacterial strains as ICC*25 was closely related to *Azospirillum brasilense* Sp7. Strain VCC*11 was in the same group as *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13 and *Azotobacter* sp. but not related to *Beijerinckia* sp. (Fig. 120).

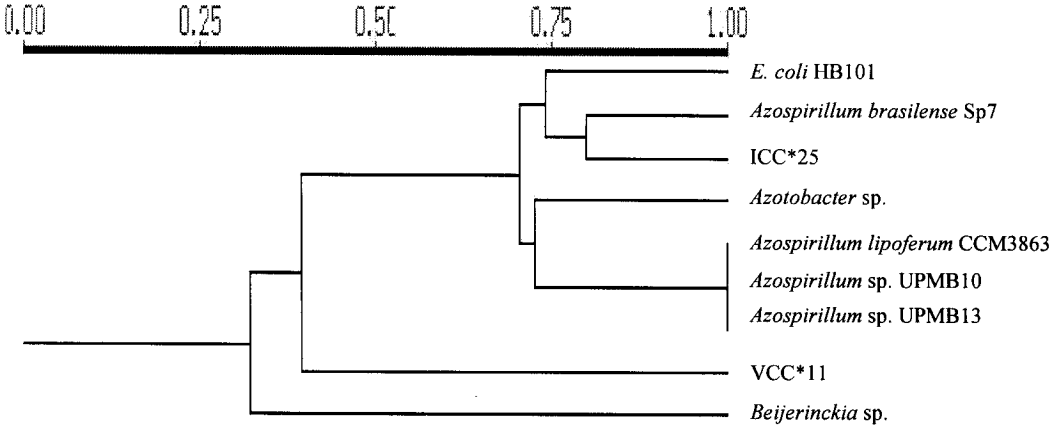

Figure 120. Dendrogram of group 35 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 36 was displayed that stain as IVNEC*11 was in the same group as *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13 and *Azotobacter* sp. but not related to *Beijerinckia* sp. (Fig. 121).

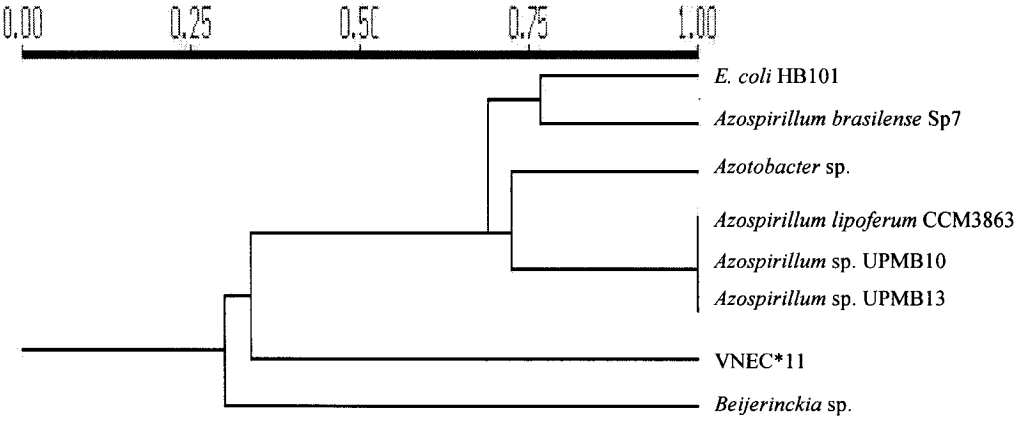

Figure 121. Dendrogram of group 35 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 37 was displayed that strain as INM324 was in the same group as *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13and *Azotobacter* sp. but not related to *Beijerinckia* sp. (Fig. 122)

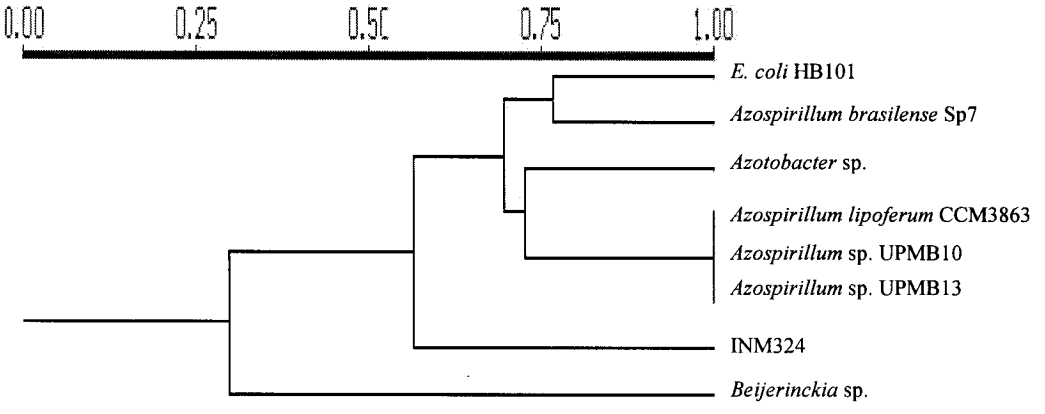

Figure 122. Dendrogram of group 37 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 38 was displayed that stain as ICC42 was related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. and VCC*22, VND31, IVNEM31, INEC*23, INM336 and INEM216 strains were not related to reference strains (Fig. 123).

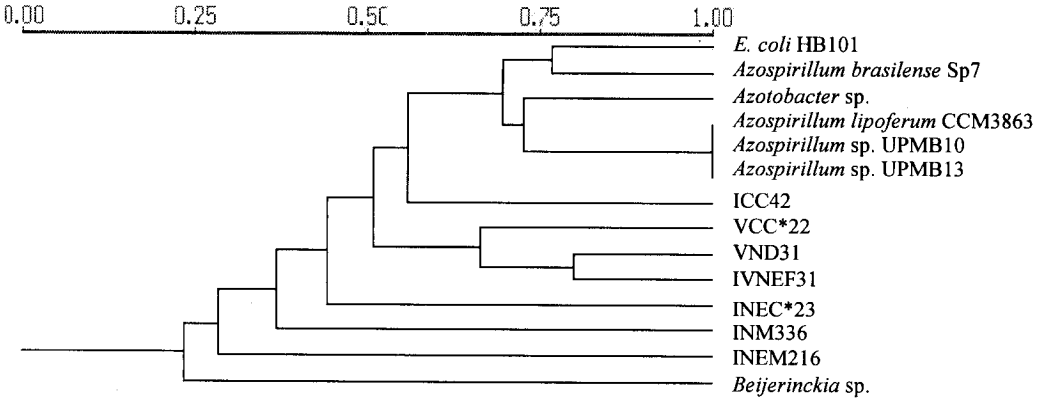

Figure 123. Dendrogram of group 38 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 39 was indicated that INF16 strain was more related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13 than strain as INC45 . Isolated strains as ICA23, ICC25 and IVNR14 were not related to *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13, *Beijerinckia* sp. and *Azotobacter* sp. (Fig. 124).

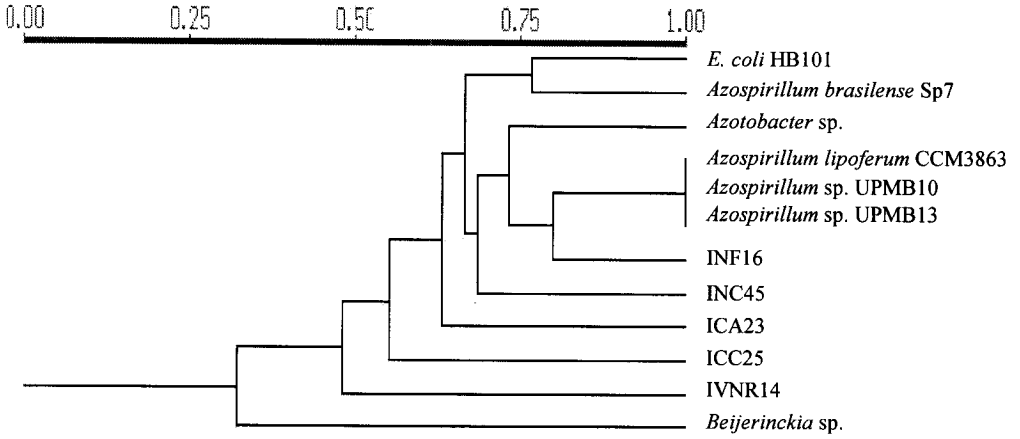

Figure 124. Dendrogram of group 39 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 40 was displayed that stain as IVCM144 was more related to *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13, *Beijerinckia* sp. and *Azotobacter* sp. than strain as IVNER11 (Fig. 125).

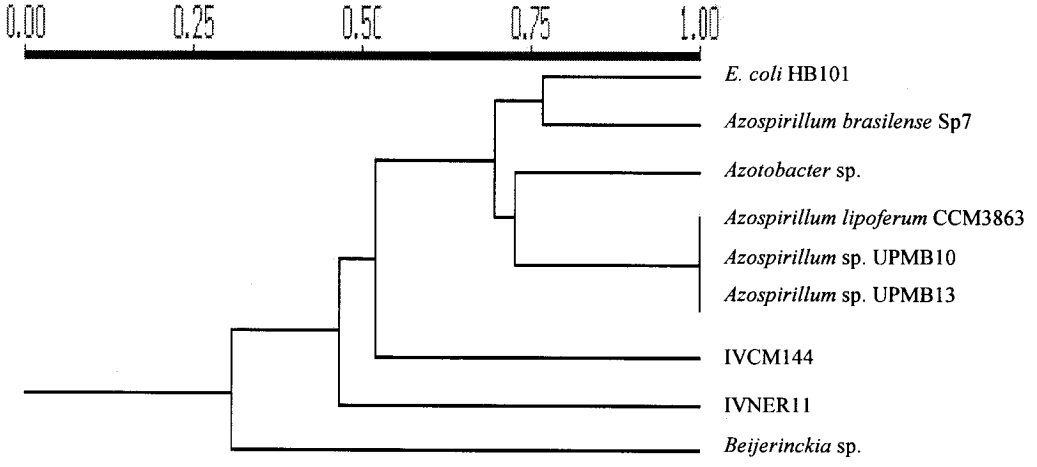

Figure 125. Dendrogram of group 40 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram in group 41 found that VNA23 strain was related to *Azotobacter* sp. Strain IVNER31 was not closely related to reference strains and strain IVNEM141 was different to reference clade (Fig. 126).

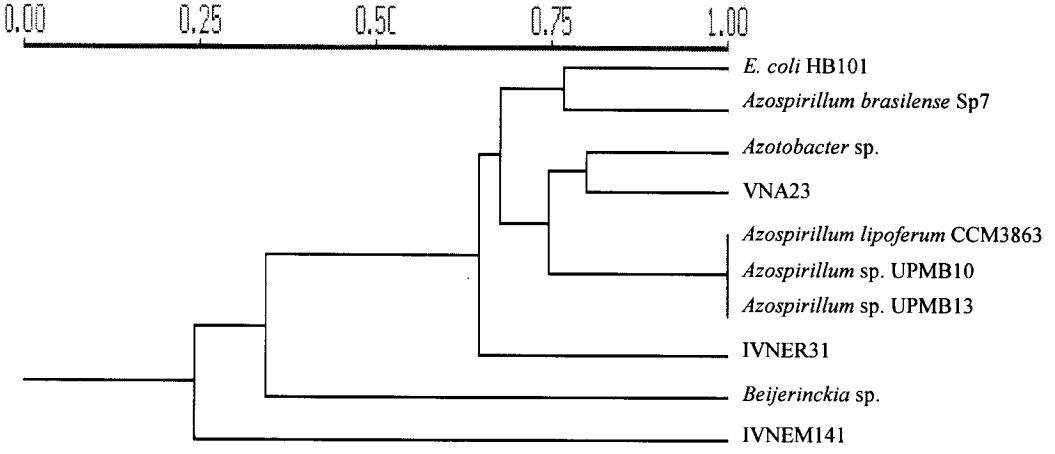

Figure 126. Dendrogram of group 41 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 42 was observed that bacterial strain as INF32 was more related to *Azotobacter* sp. than strains as VNEA21, and INM343. The strains as INEF14 and INECR11 were related to *Azospirillum lipoferum* CCM3863, *Azospirillum* sp. UPMB10 and *Azospirillum* sp. UPMB13. For bacterial srrain as VND13 was not related to reference strains (Fig. 127).

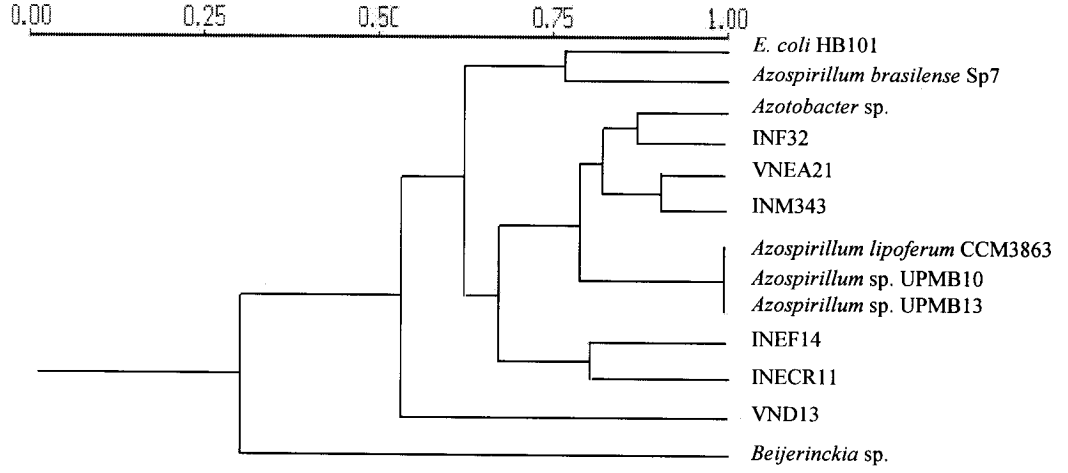

Figure 127. Dendrogram of group 42 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 43 was displayed that stain as IVNEM121 was related to *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13 and *Azotobacter* sp. (Fig. 128).

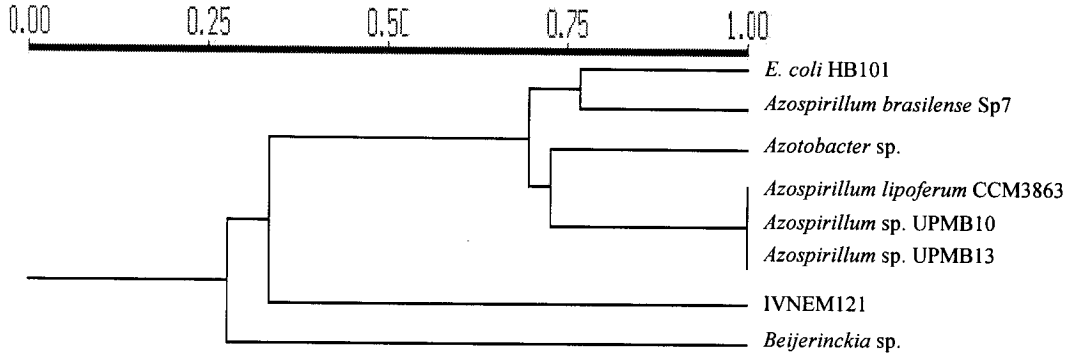

Figure 128. Dendrogram of group 43 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 44 was observed that bacterial strains as IVNM232 and IVCM222 were related to *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13 and *Azotobacter* sp.. INR12 strain was related to *Beijerinchia* sp. (Fig. 129).

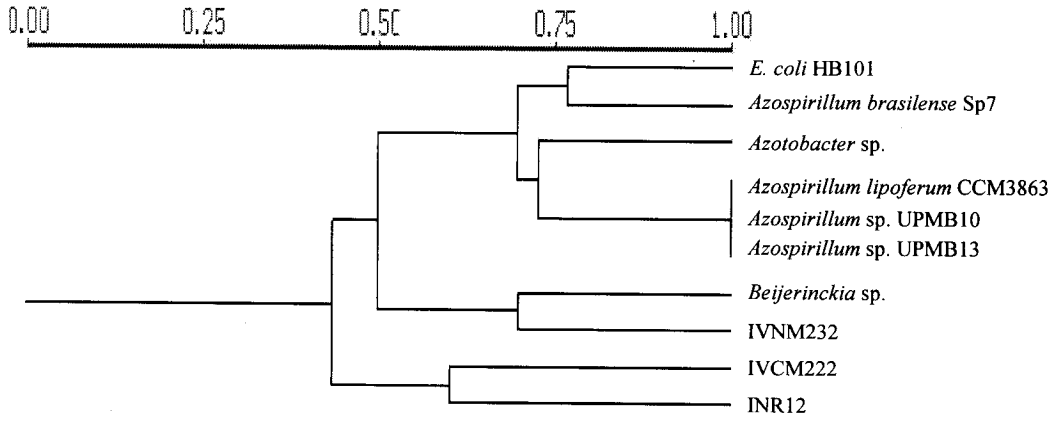

Figure 129. Dendrogram of group 44 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 45 was found that IVNM134 strain was related to *Azotobacter* sp. and strain as IVNM125 was not closed to reference strains. The strains as IVNM142 and IVCM113 were not related to *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13, *Beijerinckia* sp. and *Azotobacter* sp. (Fig.130).

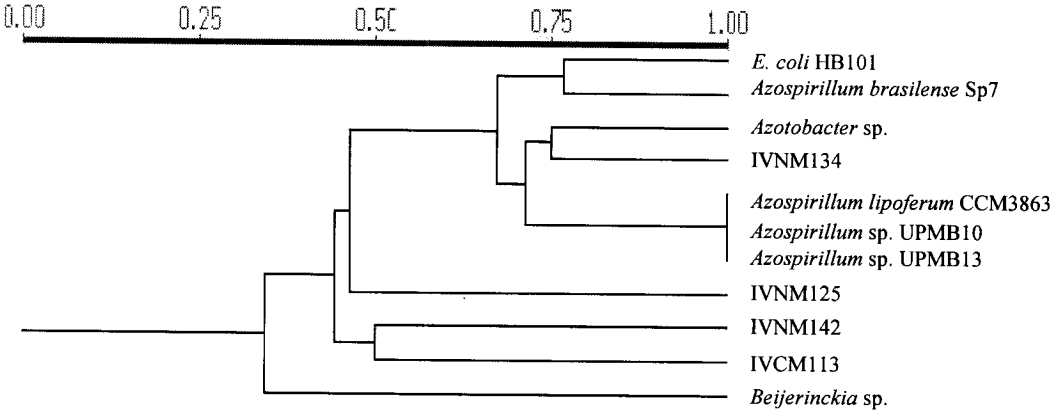

Figure 130. Dendrogram of group 45 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 46 was displayed that stain as IVCR12 was more related to *E. coli* HB101, *Azospirillum brasilenese* Sp7, *A. lipoferum* CCM3863, *Azospirillum* sp. UPMB10, *Azospirillum* sp. UPMB13 and *Azotobacter* sp. than strain IVCM131 (Fig. 131).

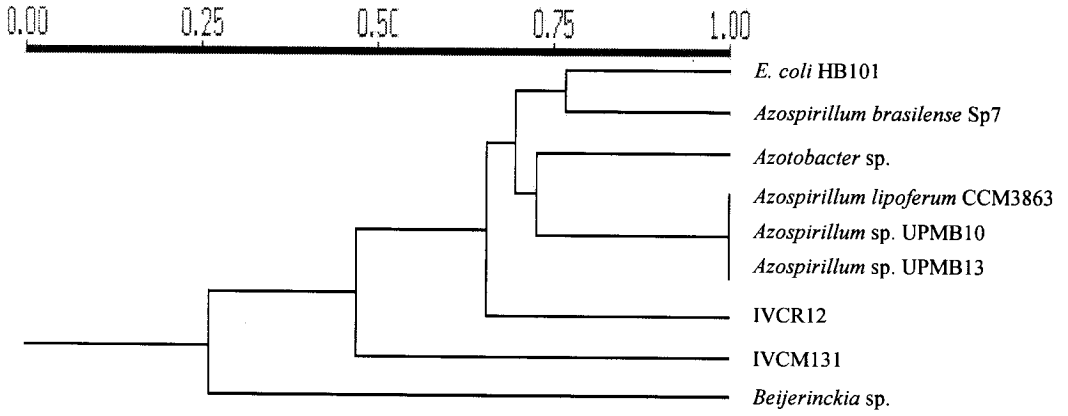

Figure 131. Dendrogram of group 46 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 47 was found that isolated strain as IVCCR14 was belong in an another different group (Fig. 132).

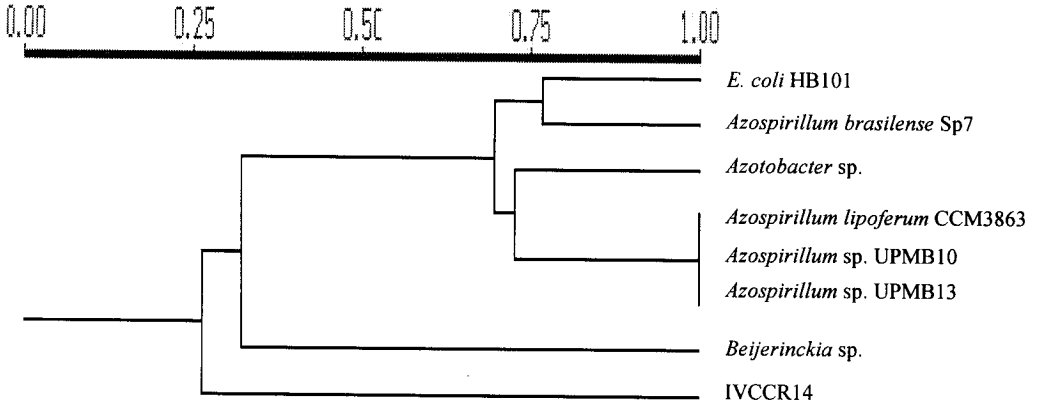

Figure 132. Dendrogram of group 47 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

Dendrogram of group 48 was found that isolated strain as IVCM123 was belong in an another different group (Fig. 133).

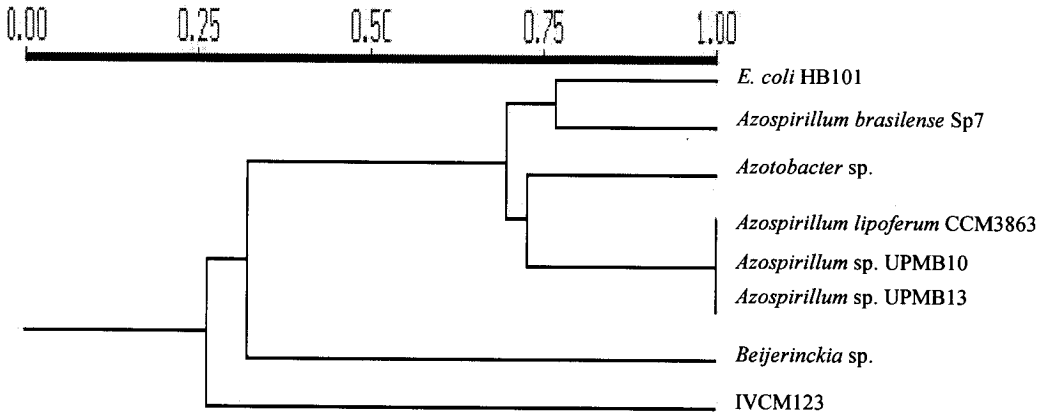

Figure 133. Dendrogram of group 48 separated *nif* D gene pattern groups of free-living nitrogen fixing bacterial isolates by using ERIC primer

CHAPTER IV

DISCUSSIONS

The diversity of bacterial isolates in thesis were studied by various methods. The first one was used morphological method and found that 222 free-living nitrogen fixing bacterial isolates were gram negative rod shape and short-rod. The result was agreed with Chocnhahirum (1986) who found that 259 bacterial cultures isolated from acid soil of Thailand were gram negative rod shape. Another study on the physiological diversity of rhizoshere *Spartina alternitlona* (smoot cord grass) in salt marsh in North America was done by Bagwell et al., (1998) and they found that all 339 strains gram negative. The majority of strains were motile rods which were confirmed gram negative of free-living nitrogen fixing bacterial.

Effectiveness of N_2-fixation was determined by acetylene reduction assay (ARA). It was found that all bacterial isolates could fix nitrogen. The N_2–fixation efficiency of free-living nitrogen fixing bacterial isolates from divered ecosystem in Thailand were also diversed ranging from 1-9,500 nmol C_2H_4/mg protein/day. The result indicated diversity of free-living nitrogen fixing bacteria. Siripin (1986) studied bacteria from the sugarcane roots and found that they were bacteria in several genus which were different in N_2-fixation efficiency. Chocnhahirum (1986) isolated bacteria from rice roots and also found that there were different N_2 –fixation.

Identification by biochemical assay found that the technique could identify only 56 isolates from 222 isolates by comparing with the reference strains such as *Beijerinckia* sp., *Azomonas* sp., *Klebsiella* sp., *Azotobacter* sp. and *Azospirillum* sp. Chocnhahirum (1986) studied chemotoxomy of some nitrogen–fixing bacteria isolated from the rhizoshere of rice (*Oryza sativa L.*) grown in Thailand. The strains as R15, R17 and R25 were isolated from of rice roots and rhizosphere soils of rice by comparing with the reference strains such as *Klebsiella oxytoc, Pseudomonas* sp. and *Azospirillum lipoferum.* The results found that the strains as R15 and R17 were similar to *Klebsiella oxytoca.* The strain as R25 was similar to *Azospirillum* sp.. Biochemical assay in this thesis could not cover identification of free-living nitrogen fixing bacteria isolates from various areas in Thailand. Because bacterial strains were isolated from the various diversities of environment and ecosystem in soils. In study of biochemical assay found that the free-living

nitrogen fixing bacterial groups were calculated that *Beijerinckia* sp. was distributed in almost regions and ecosystem systems except the Central region was found in the field crop area. The results indicated that *Beijerinckia* sp. was lived every areas specific areas. Siripin (1986) reported that *Beijerinckia* sp. was selected from the sugarcane roots. For *Klebsiella* sp. was not found in the North region but found rice in rotation with the crops area in the Central region. The North Eastern region found foot hill mountain area. *Azotobacter* sp. was found intensive agricultural production using high rate of pesticides and fertilizers. *Azomonas* sp. was low diversity in the North Eastern when comparison with *Beijerinckia* sp. but found almost ecosystems in the North and the Central regions. For *Azospirillum* sp. group was found that the low diversity in the North Eastern region but found in the North and the Central regions. The results showed that the moisture was effect metabolism of *Azospirillum* sp. example the rice area found *Azospirillum* sp. in rhizoshere and rhizoplane (Watanabe et al., 1997). Biochemical identification assays were not related to the results obtained by using *nif*D–PCR. Because biochemical identification assay using phenotypic similarity and some physiological characterization mistook for classical test of bacterial isolates. Hruek et al., (1997) and Kirchhof et al., (1996) supported that the identification of isolates was difficult if phenotypical and physiological characterization were not fully identical with a described species.

To solve this problem a rapid and accurate approach for identification of free-living nitrogen fixing bacteria at the species level was investigated. PCR-generated genemic fingerprints appeared to be the method of choice for such studies. PCR protocols which yielded the best results in molecular characterization of the genus were than used for identification of isolates from several habitat, and the results were compared with those of molecular phylogenetic analysis. *nif* D gene profile analysis was used to analyze the genetic diversity that because nitrogenase know as the key enzyme in N_2-fixing bacteria.

The *nif*D gene, encoding the alpha subunit of dinitrogenase, was one of the most informative genes among *nif* genes (Young, 1993). Another report indicated that characterization of N_2-fixing bacterial communities in rice roots by analysis of *nif*D sequences could be done directly from a total DNA extract (Ueda et al., 1995). In this study by using *nif*D-PCR gene product bacterial isolates from 222 isolates obtained from 11 areas in different 48 groups.

The UPGMA method was used to analyze the *nif*D variation within free-living bacterial population. The result obtained from this study showed that the majority of genetic diversity of bacterial isolates were related between each area and region. Dendrogram constructed from *nif*D-PCR product patterns could separate free-living nitrogen fixing bacterial isolates into 4 groups which were classified as similar strains, dominant native strains, closed related to reference strains and other different strains. For the dominant native strains of free-living nitrogen fixing bacteria isolated from different soils in Thailand it were found in the areas as highest mountain in North region, middle mountain in North region, rice in rotation with other crops in Central region, uncultivated in North Eastern region, undisturbed forest in North region and forest clearance for crop cultivation for 3 years in North region. The biodiversity of bacterial isolates from this study found that some areas as the mountain, undisturb forest and forest clearance for crop cultivation for 3 years area showed high diversity among bacteria group. These data indicated the influence by undisturb ecosystem to bacterial diversity in natural areas. Forest clearance for crop cultivation for 3 years area showed high effect by agricultural system. The other area which found similar strains and dominant native strains could explain that the influence by agricultural system was controlled bacterial diversity in soils.

The identification of free-living nitrogen fixing bacterial strains were *nif*D-PCR product groups were separated by ERIC-PCR primer. Because all bacterial strains were gram negative that ERIC primer could detect in large variety of bacteria specific gram negative bacteria. Frans (1992) supported that ERIC primer as specific oligonucleotide primer the genomes of a variety of eubacteria specific gram negative bacteria. The information reported by Versalovic et al., (1991) ERIC-PCR could became a powerful tool for the molecular genetic analysis of bacteria and for bacterial taxonomy, since it allows the fingerprinting of individual genera, species and strains. In study ERIC-PCR analysis could divide 48 groups *nif*D-PCR product groups that ERIC-PCR analysis separated specific characteristic for diversity of free-living nitrogen fixing bacterial group such as related to reference strains, not related to reference strains, closed to reference strains, not closed to reference strains and another different groups. The results indicated that ERIC-PCR analysis could be used separately to generate the fingerprinting characteristic of strains. Using ERIC primer found that free-living nitrogen fixing bacteria were high diversity and were not found specific relation with each ecosystem when determined from ERIC-PCR products. Kirchief

et al., (1997) observed that fingerprinting techniques using ERIC-PCR was able to determine the diversity of population at strain level. Thus this technique could be successfully used to differentiate strains of free-living nitrogen fixing bacteria.

The free-living nitrogen fixing bacterial strain in field crop cultivation area from the Central region as ICC11 was interesting because bacterial strain as ICC11 had the highest effectiveness of N_2-fixation. Strain ICC11 was white of colony and negative short-rod shape. To identify of biochemical assay the free-living nitrogen fixing bacterial strain as ICC11 was found that the results could not identify. For studying in genetic level by *nif*D-PCR patterns of bacterial strain as ICC11 was represented group 4 and ICC11 was found that closely related to *Beijerinckia* sp., *Azotobacter* sp. and *Azospirillum brasilenese* Sp7. When strain ICC11 was used by ERIC primer, it found that strain ICC11 was more related to *Azotobacter* sp. than other. The results indicated that strain ICC11 was found closely related to *Azotobacter* sp.. In studying strain ICC11 was brought for increasing soil fertility in sustainable agriculture and forestry.

CHAPTER V

CONCLUSIONS

The objective of this study was to investigate biodiversity of free-living nitrogen fixing bacteria isolated in Thailand with regards to diversification in genetic level and in conjunction with population changes in each ecosystem. This might be able to bring bout the management for proper utilization of free-living nitrogen fixing bacteria in increasing soil fertility in sustainable agriculture and forestry. Site selection was made in three regions; the North, the Northeast and the Central.

The result obtained from this research were sumarized as followings:

1. The study on morphology of free-living nitrogen fixing bacteria was found that 222 isolates were gram negative rod shape accounted for 63.51% and gram negative short-rod shape 36.49 %.

2. The study on physiology of free-living nitrogen fixing bacteria was found that effectiveness in N_2-fixation were wide range in N_2-fixing efficiency (1-9,500 nmol C_2H_4/mg protein/day).

3. Biochemical assay could identify 56 isolates as following; *Beijerinckia* sp. 16 isolates, *Klebsiella* sp. 4 isolates, *Azotobacter* sp. 1 isolate, *Azomonas* sp. 18 isolates and *Azospirillum* sp. 17 isolates.

4. The use of *nif*D-PCR patterns could separate into different 48 groups and dendrogram of *nif*D-PCR product patterns could divide free-living nitrogen fixing bacterial isolates into 4 groups which were classified as similar strains, dominant native strains, closed related to reference strains and other different strains.

5. The use of ERIC-PCR from dendrogram could explain the diversity of population at strain level and differentiate strains of free-living nitrogen fixing bacteria.

6. Free-living nitrogen fixing bacterial strain from soils in Thailand were high diversity in terms of species. The free-living nitrogen fixing bacteria were not specific related with each ecosystem.

The results from this thesis could be used for further studies.

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APPENDIX A

Table 1. Characterestic of free-living mitrcgen fixing becteria isolates in each area

Isolate	Media	Morphology					ARA (nmol/mg/ day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
INM111	M	circular	white	0.3	⊖	0.45x3.84	979.71	-	-	+	-	-	+	-	white
INM125	CCM	circular	white	0.06	⊖	0.88x1.44	830.00	-	-	-	-	-	+	-	white
INM133	CCM	circular	white	0.4	⊖	0.48x1.92	354.23	+	-	-	-	-	+	-	white
INM145	M	circular	clear	0.2	⊖	1.15x1.21	912.85	-	-	+	-	-	+	-	clear
IVNM116	M	circular	orage	0.05	⊖	0.96x1.15	12.80	-	-	+	-	-	+	-	orage
IVNM125	S	circular	pink	0.05	⊖	0.46x0.96	143.33	-	-	-	-	+	+	-	pink
IVNM134	S	circular	pink	0.1	⊖	0.46x0.96	20.00	+	+	-	+	-	+	+	pink
IVNM142	S	circular	pink	0.1	⊖	0.46x1.15	53.00	-	-	-	-	-	+	+	pink
INM213	G	circular	white	0.1	⊖	0.80x1.5	3.75	-	-	-	-	-	+	-	white
INM224	G	circular	clear	0.1	⊖	0.48x0.57	134.50	+	-	-	+	-	+	-	clear
INM236	S	circular	clear	0.1	⊖	0.48x0.96	187.50	-	-	-	-	-	+	-	clear
INM248	CCM	circular	white	0.1	⊖	0.96x1.92	312.10	-	-	-	-	-	+	-	white
IVNM212	S	circular	pink	0.1	⊖	0.96x1.15	106.36	-	-	-	-	-	+	-	pink
IVNM224	M	circular	pink	0.05	⊖	0.96x1.92	42.50	+	+	+	-	-	+	+	pink

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/ day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
IVNM232	S	circular	clear	0.1	⊖	0.46x0.96	36.13	-	-	-	-	-	+	+	clear
IVNM243	S	circular	pink	0.05	⊖	0.76x1.15	35.60	-	+	-	-	-	+	+	pink
INM314	M	circular	white	0.1	⊖	0.48x0.96	253.20	-	-	+	-	-	+	-	white
INM324	G	circular	white	0.2	⊖	0.96x1.44	451.20	-	-	-	-	-	+	-	white
INM336	M	circular	clear	0.1	⊖	0.48x0.96	16.71	+	-	+	-	-	+	-	clear
INM343	CCM	circular	clear	0.01	⊖	0.96x2.86	145.31	-	-	-	-	-	+	-	clear
IVNM311	S	circular	white	0.02	⊖	0.46x0.96	170.00	-	-	-	-	-	+	-	clear
IVNM322	S	circular	white	0.02	⊖	0.96x1.44	67.80	-	-	-	-	-	+	-	white
IVNM331	S	circular	white	0.01	⊖	0.96x1.92	24.50	-	+	-	-	-	+	+	white
IVNM341	S	circular	white	0.05	⊖	0.96x1.92	60.60	-	+	-	-	-	+	+	white
INCR11	G	circular	clear	0.2	⊖	0.96x1.92	181.73	-	-	-	-	-	+	-	clear
INCR24	G	circular	clear	0.1	⊖	0.48x1.44	834.23	-	-	-	-	-	+	-	clear
INCR36	M	circular	clear	0.2	⊖	0.81x1.50	521.60	-	-	+	+	-	+	-	clear
INCR410	CCM	circular	white	0.1	⊖	0.48x1.92	2292.50	-	-	-	-	-	+	-	white
IVNCR15	M	circular	white	0.1	⊖	0.92x1.92	21.80	-	+	+	+	-	+	+	white

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/ day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
IVNCR22	S	circular	yellow	0.1	⊖	0.92x1.92	57.00	-	+	-	-	-	+	+	yellow
IVNCR34	M	circular	pink	0.1	⊖	0.48x0.76	64.30	-	+	+	+	-	+	+	pink
IVNCR44	S	circular	white	0.05	⊖	1.05x1.92	23.10	-	+	-	-	-	+	+	white
INR12	S	circular	white	0.1	⊖	0.96x1.92	117.39	-	+	-	-	+	+	-	white
INR25	CCM	circular	white	0.05	⊖	0.96x1.44	486.00	+	-	-	-	-	+	-	white
INR33	S	circular	clear	0.1	⊖	0.48x1.12	600.00	-	-	-	-	-	+	-	clear
INR43	G	circular	clear	0.2	⊖	0.96x2.40	329.37	-	-	-	-	-	+	-	clear
IVNR13	M	circular	yellow	0.15	⊖	0.48x0.50	9.00	+	+	+	-	-	+	+	yellow
IVNR26	M	circular	clear	0.2	⊖	0.48x0.92	30.00	-	+	-	+	-	+	+	clear
IVNR32	S	circular	pink	0.1	⊖	0.96x1.48	150.00	+	+	-	-	-	+	+	pink
IVNR44	M	circular	white	0.1	⊖	0.96x1.96	175.11	-	+	+	-	-	+	+	white
INC15	G	circular	white	0.2	⊖	0.48x1.12	600.00	-	-	-	-	-	+	-	white
INC29	M	circular	white	0.1	⊖	0.96x2.88	93.20	-	-	+	-	-	+	-	white
INC31	G	circular	white	0.3	⊖	0.96x1.48	150.00	-	-	-	+	-	+	-	white
INC45	G	circular	white	0.15	⊖	0.96x2.10	167.36	-	-	-	-	-	+	-	white

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/ day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
IVNC13	S	circular	white	0.1	⊖	0.48x0.96	155.60	-	-	-	-	-	+	+	white
IVNC24	S	circular	white	0.05	⊖	0.96x1.44	135.04	-	+	-	-	-	+	+	white
IVNC31	S	circular	white	0.1	⊖	0.96x1.44	22.50	-	+	-	-	-	+	+	white
IVNC41	S	circular	yellow	0.15	⊖	0.96x1.96	68.10	+	-	-	-	-	+	-	yellow
INF16	M	circular	pink	0.01	⊖	0.96x1.92	20.00	-	-	+	-	-	+	-	pink
INF25	S	circular	white	0.2	⊖	0.77x0.96	74.78	+	-	-	-	-	+	-	white
INF32	S	circular	clear	0.1	⊖	0.45x0.76	8.31	-	-	-	-	-	+	-	clear
INF47	S	circular	white	0.1	⊖	0.96x1.44	43.65	-	+	-	-	-	+	-	white
IVNF11	S	circular	clear	0.2	⊖	0.48x0.96	1.34	-	-	-	-	-	+	-	clear
IVNF24	S	circular	white	0.08	⊖	0.96x1.96	27.50	+	+	-	-	-	+	+	white
IVNF31	S	circular	clear	0.4	⊖	0.48x1.44	202.90	-	-	-	+	-	+	+	clear
IVNF41	S	circular	white	0.05	⊖	0.96x1.96	69.00	+	-	-	-	-	+	+	white
IND13	G	circular	white	0.1	⊖	0.288x1.24	118.00	-	-	-	-	-	+	-	white
IND24	S	circular	white	0.3	⊖	0.30x0.48	329.80	-	-	-	-	-	+	-	white
IND39	CCM	circular	white	0.1	⊖	0.96x1.92	1266.00	+	-	-	-	-	+	-	white
VND13	S	circular	white	0.09	⊖	0.96x1.44	7.15	-	-	-	-	-	+	+	white

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/ day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
VND26	S	circular	white	0.1	⊖	0.48x0.96	41.50	-	-	-	-	-	+	+	white
VND31	S	circular	clear	0.1	⊖	0.48x0.96	87.50	-	+	-	-	-	+	-	clear
INA19	S	circular	white	0.1	⊖	0.96x1.92	1266.00	+	-	-	-	-	+	-	white
INA23	G	circular	white	0.1	⊖	0.96x1.92	2345.25	-	-	-	-	-	+	-	white
VNA13	S	circular	clear	0.15	⊖	0.86x0.96	220.00	-	+	-	-	-	+	-	white
VNA23	S	circular	clear	0.1	⊖	0.48x1.15	48.33	-	+	-	-	-	+	+	clear
INB13	G	circular	white	0.2	⊖	0.57x0.96	76.48	+	-	-	-	-	+	-	white
INB24	G	circular	white	0.02	⊖	0.96x1.19	329.37	-	+	-	-	-	+	-	white
VNB14	M	circular	white	0.05	⊖	0.48x0.96	81.68	-	-	+	-	-	+	-	white
VNB21	S	circular	yellow	0.01	⊖	0.96x1.44	9.00	-	+	-	-	-	+	+	yellow
INC*16	S	circular	clear	0.1	⊖	0.81x0.96	468.50	-	-	-	-	-	+	-	clear
INC*28	M	circular	clear	0.2	⊖	0.28x0.96	188.42	-	-	-	-	-	+	-	clear
VNC*12	S	circular	clear	0.01	⊖	0.96x1.72	3.37	-	-	+	+	-	+	-	clear
VNC*24	M	circular	white	0.02	⊖	0.96x1.15	13.40	-	+	+	+	-	+	+	white
ICM118	M	circular	clear	0.1	⊖	0.48x0.96	43.49	-	-	+	-	-	+	-	clear
ICM123	G	circular	white	0.1	⊖	0.48x0.96	50.00	+	+	-	-	-	+	-	white
ICM137	M	circular	white	0.01	⊖	0.96x1.96	135.00	-	-	+	-	-	+	-	white

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/ day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
ICM147	M	circular	white	0.09	⊖	0.76x0.96	143.00	-	-	+	-	-	+	-	white
IVCM113	S	circular	yellow	0.1	⊖	0.57x1.15	158.57	+	+	-	-	-	+	+	yellow
IVCM123	M	circular	white	0.08	⊖	0.96x1.96	1.20	-	+	+	-	-	+	+	white
IVCM131	S	circular	pink	0.1	⊖	0.38x1.15	72.18	-	-	-	-	-	+	+	pink
IVCM144	M	circular	pink	0.09	⊖	0.48x0.96	49.50	+	-	+	-	-	+	+	pink
ICM219	S	circular	white	0.1	⊖	0.86x1.44	159.93	+	+	-	-	-	+	-	white
ICM222	G	circular	clear	0.1	⊖	0.74x0.87	594.90	-	-	-	-	-	+	-	clear
ICM235	S	circular	white	0.01	⊖	0.48x1.44	34.70	-	-	-	-	-	+	-	clear
ICM249	M	circular	yellow	0.1	⊖	0.76x0.96	196.93	-	-	+	-	-	+	-	yellow
IVCM213	M	circular	white	0.05	⊖	0.96x1.96	55.43	+	+	+	-	-	+	+	white
IVCM222	M	circular	white	0.08	⊖	0.96x1.96	117.50	-	-	+	-	-	+	-	white
IVCM233	M	circular	clear	0.1	⊖	0.48x1.15	272.31	-	-	+	-	-	+	+	clear
IVCM243	S	circular	white	0.2	⊖	0.96x1.44	28.30	-	-	-	-	+	+	-	white
ICM312	M	circular	white	0.09	⊖	0.96x1.96	251.43	-	-	+	-	-	+	-	white
ICM321	G	circular	clear	0.3	⊖	0.48x1.92	343.50	-	-	-	-	-	+	-	clear
ICM332	G	circular	white	0.1	⊖	0.96x1.92	240.00	+	+	-	-	-	+	+	white

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
ICM347	M	circular	yellow	0.1	⊖	0.86x0.96	392.00	-	+	+	-	-	+	+	yellow
IVCM315	M	circular	pink	0.1	⊖	0.76x1.96	1.38	-	-	+	-	-	+	+	pink
IVCM323	S	circular	clear	0.07	⊖	0.48x0.96	82.50	-	+	-	+	-	+	+	clear
IVCM335	S	circular	white	0.01	⊖	0.48x0.57	20.30	-	+	-	-	-	+	-	pink
IVCM341	S	circular	clear	0.1	⊖	0.96x1.96	45.00	-	-	-	-	-	+	-	clear
ICCR16	M	circular	yellow	0.1	⊖	0.76x1.92	6.15	-	-	+	-	-	+	-	yellow
ICCR26	M	circular	white	0.01	⊖	0.48x1.96	176.88	-	-	+	-	-	+	-	white
ICCR38	M	circular	white	0.2	⊖	0.96x1.92	198.28	-	-	+	-	-	+	-	white
ICCR41	S	circular	white	0.01	⊖	0.96x1.92	4144.00	-	+	-	+	-	+	+	white
IVCCR14	S	circular	clear	0.1	⊖	0.48x0.96	36.66	+	+	-	+	-	+	+	white
IVCCR21	S	circular	clear	0.1	⊖	0.64x1.96	36.80	-	+	-	-	+	+	+	clear
IVCCR31	S	circular	clear	0.1	⊖	0.74x1.11	25.20	+	+	-	+	-	+	+	white
IVCCR41	S	circular	clear	0.1	⊖	0.35x0.96	86.04	-	+	-	-	-	+	+	clear
ICR12	G	circular	white	0.01	⊖	0.96x1.96	228.40	-	-	-	+	-	+	-	white
ICR24	S	circular	white	0.03	⊖	0.96x2.40	8.40	-	-	-	+	-	+	-	white
ICR35	M	circular	white	0.01	⊖	0.96x1.78	3273.30	-	+	-	-	-	+	-	white

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
ICR44	S	circular	white	0.1	⊖	0.96x1.92	4144.00	+	-	-	-	-	+	-	white
IVCR12	S	circular	pink	0.03	⊖	0.28x0.48	64.00	+	-	-	-	-	+	-	pink
IVCR21	M	circular	clear	0.1	⊖	0.48x0.96	16.60	+	-	+	-	-	+	-	clear
IVCR33	M	circular	white	0.1	⊖	0.96x1.15	19.71	-	+	+	-	-	+	+	white
IVCR41	S	circular	clear	0.08	⊖	0.96x1.96	78.70	-	-	-	-	-	+	-	clear
ICC11	G	circular	white	0.3	⊖	0.48x0.54	9300.00	-	+	-	-	-	+	-	white
ICC25	S	circular	clear	0.1	⊖	0.19x0.29	1.80	-	+	-	-	-	+	+	clear
ICC34	S	circular	white	0.1	⊖	0.77x0.96	211.45	-	+	-	-	-	+	-	white
ICC42	G	circular	white	0.07	⊖	0.48x1.92	197.50	+	+	-	+	-	+	+	white
IVCC13	M	circular	pink	0.1	⊖	0.96x1.05	11.25	-	+	-	-	-	+	+	pink
IVCC24	M	circular	white	0.01	⊖	0.96x1.96	28.50	-	+	+	-	-	+	+	white
IVCC32	S	circular	white	0.02	⊖	0.96x1.15	45.00	-	-	-	-	-	+	+	white
IVCC45	S	circular	pink	0.08	⊖	0.48x2.88	45.00	-	+	-	-	-	+	+	pink
ICF15	M	circular	white	0.2	⊖	0.76x1.15	1134.33	-	-	+	-	-	+	-	white
ICF24	S	circular	white	0.2	⊖	0.96x2.40	101.20	-	-	-	-	-	+	-	white
ICF39	M	circular	white	0.05	⊖	0.96x1.92	171.00	-	-	+	-	-	+	-	white

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/ day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
ICF43	G	circular	clear	0.05	⊖	1.44x1.92	3292.00	-	-	-	-	-	+	-	clear
IVCF13	M	circular	clear	0.1	⊖	0.96x1.92	7.00	-	+	+	-	-	+	+	clear
IVCF24	S	circular	pink	0.1	⊖	0.48x0.96	7.25	-	+	-	-	-	+	+	pink
IVCF32	S	circular	white	0.05	⊖	0.48x0.96	39.70	+	+	-	-	-	+	+	white
IVCF42	M	circular	white	0.05	⊖	0.48x0.96	8.20	-	+	+	-	-	+	+	white
ICD13	S	circular	white	0.09	⊖	0.54x0.79	925.10	-	-	-	-	-	+	-	white
ICD21	S	circular	pink	0.01	⊖	0.76x1.72	92.00	-	-	-	-	-	+	-	pink
ICD31	G	circular	white	0.08	⊖	0.28x0.48	589.66	-	-	-	-	-	+	-	white
VCD11	S	circular	white	0.05	⊖	0.981x1.92	115.38	-	-	-	+	-	+	+	white
VCD21	S	circular	white	0.1	⊖	0.981x1.92	9.27	-	-	-	-	-	+	+	white
VCD31	M	circular	clear	0.05	⊖	0.48x0.96	3.60	-	-	+	-	-	+	-	white
ICA16	M	circular	white	0.02	⊖	0.96x1.92	89.62	-	+	+	-	-	+	-	white
ICA23	M	circular	white	0.01	⊖	0.84x1.92	21.42	+	-	-	+	-	+	-	white
VCA12	S	circular	clear	0.05	⊖	0.76x0.96	37.22	-	-	-	-	-	+	-	clear
VCA22	S	circular	orage	0.1	⊖	0.48x1.92	2.82	-	-	-	-	-	+	-	orage
ICB12	G	circular	white	0.1	⊖	0.96x1.92	673.07	-	-	-	-	-	+	-	white

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/ day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
ICB27	M	circular	clear	0.1	⊖	0.96x1.17	14.34	+	-	+	-	-	+	+	white
VCB11	S	circular	white	0.1	⊖	0.48x1.92	253.84	-	-	-	-	-	+	-	white
VCB23	S	circular	yellow	0.01	⊖	0.96x1.92	28.50	-	-	-	+	-	+	-	yellow
ICC*13	S	circular	white	0.01	⊖	0.96x1.92	413.80	-	-	-	-	-	+	-	white
ICC*25	S	circular	white	0.35	⊖	0.96x1.92	414.93	-	+	-	-	-	+	+	white
VCC*11	S	circular	clear	0.1	⊖	0.48x0.96	109.09	-	-	-	+	-	+	+	clear
VCC*22	S	circular	clear	0.3	⊖	0.96x1.92	12.22	-	-	-	-	-	+	-	clear
INEM114	S	circular	white	0.01	⊖	0.76x1.46	394.22	+	+	-	-	-	+	+	white
INEM123	S	circular	white	0.2	⊖	0.96x1.96	470.83	-	+	-	-	-	+	+	white
INEM133	S	circular	white	0.1	⊖	0.98x1.84	102.00	-	+	-	-	-	+	+	white
INEM143	S	circular	clear	0.5	⊖	0.48x1.14	57.00	+	+	-	-	-	+	+	clear
IVNEM112	S	circular	white	0.1	⊖	0.48x1.96	75.00	-	+	-	-	-	+	+	white
IVNEM121	S	circular	white	0.1	⊖	0.48x0.96	66.46	-	-	-	-	-	+	+	white
IVNEM133	S	circular	white	0.05	⊖	0.76x0.96	69.40	+	+	-	+	-	+	+	white
IVNEM141	M	circular	white	0.1	⊖	0.76x1.05	76.90	+	+	+	-	-	+	+	white
INEM216	M	circular	white	0.01	⊖	0.98x1.84	48.64	-	+	+	-	-	+	+	white

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/ day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
INEM223	S	circular	white	0.05	⊖	0.98x1.84	52.00	-	-	-	-	-	+	+	white
INEM236	M	circular	white	0.02	⊖	0.79x1.96	242.10	-	+	+	-	-	+	+	white
INEM244	S	circular	white	0.02	⊖	0.96x1.96	654.39	-	+	-	-	-	+	+	white
IVNEM211	S	circular	white	0.15	⊖	0.96x1.44	48.28	-	+	-	-	-	+	+	white
IVNEM224	S	circular	pink	0.1	⊖	0.48x0.96	540.00	-	+	-	-	-	+	+	pink
IVNEM231	S	circular	white	0.1	⊖	0.48x0.96	822.80	-	+	-	-	-	+	+	white
IVNEM241	S	circular	clear	0.05	⊖	0.57x1.72	44.68	-	-	-	-	-	+	+	clear
INEM314	S	circular	white	0.1	⊖	0.76x1.44	8.52	-	+	-	-	-	+	+	white
INEM323	S	circular	clear	0.1	⊖	0.98x1.84	95.00	-	+	-	-	-	+	+	clear
INEM332	S	circular	white	0.1	⊖	0.79x0.96	277.50	-	+	-	-	-	+	-	white
INEM345	M	circular	white	0.08	⊖	0.48x1.18	6.64	-	-	+	-	-	+	+	white
IVNEM314	S	circular	pink	0.1	⊖	0.76x0.96	106.60	+	+	-	-	-	+	+	pink
IVNEM324	M	circular	clear	0.1	⊖	0.96x1.92	57.90	-	-	+	-	-	+	+	clear
IVNEM333	S	circular	pink	0.1	⊖	0.48x0.96	6.33	+	+	-	-	-	+	+	pink
IVNEM341	S	circular	clear	0.15	⊖	0.48x0.96	39.00	-	-	-	-	-	+	-	clear
INECR11	G	circular	gray	0.05	⊖	0.96x1.96	105.89	-	+	-	-	-	+	+	gray

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/ day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
INECR22	S	circular	white	0.08	⊖	0.96x1.08	232.14	+	+	-	-	-	+	+	white
INECR37	M	circular	white	0.09	⊖	0.96x1.92	18.06	-	+	-	-	-	+	+	white
INECR43	G	circular	white	0.07	⊖	1.96x3.36	190.59	-	-	-	-	-	+	-	white
IVNECR12	S	circular	white	0.15	⊖	0.96x1.41	42.25	-	-	-	-	-	+	+	white
IVNECR22	S	circular	pink	0.1	⊖	0.96x1.24	53.57	-	-	-	+	-	+	+	pink
IVNECR33	M	circular	white	0.1	⊖	0.48x0.96	149.64	-	+	+	-	-	+	+	pink
IVNECR44	S	circular	pink	0.1	⊖	0.48x1.44	208.06	-	+	-	-	-	+	+	pink
INER15	M	circular	white	0.01	⊖	0.96x1.96	178.00	-	+	+	-	-	+	+	white
INER24	M	circular	white	0.01	⊖	0.96x1.96	7.70	-	+	+	-	-	+	+	white
INER34	M	circular	yellow	0.15	⊖	0.48x0.96	499.35	-	+	+	-	-	+	+	yellow
INER46	M	circular	white	0.07	⊖	0.48x0.96	13.00	-	+	+	-	-	+	+	white
IVNER11	S	circular	clear	0.05	⊖	0.96x1.92	126.00	-	-	-	+	+	+	+	clear
IVNER21	S	circular	white	0.15	⊖	0.48x1.92	84.10	+	+	-	-	-	+	+	white
IVNER31	S	circular	yellow	0.08	⊖	0.48x0.96	69.00	-	-	-	-	-	+	+	yellow
IVNER42	S	circular	yellow	0.1	⊖	0.29x0.48	21.17	-	+	-	+	-	+	+	yellow
INEC14	S	circular	clear	0.21	⊖	0.86x1.96	175.00	-		-	+	-	+	+	clear

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
INEC23	S	circular	white	0.01	⊖	0.28x0.47	1275.00	-	+	-	-	-	+	+	white
INEC33	G	circular	yellow	0.09	⊖	0.37x0.47	119.50	-	-	-	-	-	+	-	yellow
INEC48	M	circular	white	0.01	⊖	0.76x0.96	82.62	+	+	+	-	-	+	+	white
IVNEC12	S	circular	clear	0.2	⊖	0.48x1.48	59.16	-	+	-	-	-	+	+	clear
IVNEC22	S	circular	white	0.08	⊖	0.48x1.44	210.00	-	+	-	-	-	+	-	white
IVNEC33	S	circular	clear	0.15	⊖	0.48x1.15	17.08	-	+	-	+	-	+	+	clear
IVNEC42	S	circular	white	0.25	⊖	0.76x1.44	15.86	-	-	-	+	-	+	+	white
INEF14	M	circular	white	0.05	⊖	0.98x1.84	24.75	-	-	+	-	-	+	-	white
INEF27	M	circular	yellow	0.05	⊖	0.76x1.84	16.66	-	+	+	-	-	+	+	white
INEF33	G	circular	white	0.15	⊖	0.76x0.96	499.35	+	+	-	-	-	+	+	white
INEF47	M	circular	clear	0.1	⊖	0.48x0.96	1291.00	-	-	+	-	-	+	+	clear
IVNEF12	M	circular	clear	0.1	⊖	0.48x0.96	129.37	-	+	+	+	-	+	+	white
IVNEF24	S	circular	orage	0.1	⊖	0.92x1.92	234.00	-	-	-	-	-	+	-	orage
IVNEF31	S	circular	white	0.2	⊖	0.92x1.92	48.33	-	+	-	-	-	+	+	white

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/ day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
IVNEF42	M	circular	white	0.05	⊖	0.96x1.92	247.76	-	-	+	+	-	+	+	white
INED17	S	circular	white	0.1	⊖	0.96x1.48	1291.00	-	-	-	-	-	+	+	white
INED23	G	circular	orage	0.2	⊖	0.76x0.96	81.12	-	+	-	+	-	+	-	orage
INED33	S	circular	clear	0.01	⊖	0.48x1.96	185.30	-	+	-	+	-	+	+	clear
VNED11	S	circular	clear	0.05	⊖	0.96x1.92	21.85	+	-	-	-	-	+	-	white
VNED22	S	circular	white	0.06	⊖	0.96x1.92	77.70	-	+	-	-	-	+	+	white
VNED37	M	circular	whtie	0.05	⊖	1.44x2.48	33.00	-	+	+	-	-	+	+	whtie
INEA17	M	circular	white	0.01	⊖	0.96x1.96	469.09	-	+	+	-	-	+	+	white
INEA27	M	circular	white	0.01	⊖	0.96x1.96	345.95	-	+	+	-	-	+	+	white
VNEA11	S	circular	white	0.04	⊖	0.86x0.96	7.19	-	-	-	+	-	+	-	white
VNEA21	S	circular	white	0.06	⊖	0.96x1.92	75.00	-	+	-	+	-	+	+	white
INEB11	G	circular	white	0.1	⊖	0.96x1.92	14.70	-	+	-	+	-	+	+	white
INEB21	G	circular	clear	0.3	⊖	0.48x0.96	2.14	-	-	-	+	-	+	+	white
VNEB14	M	circular	yellow	0.08	⊖	0.48x0.96	24.53	-	+	+	-	-	+	+	yellow

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
VNEB21	S	circular	yellow	0.15	⊖	0.96x1.92	18.81	-	+	-	-	-	+	+	yellow
INEC*16	M	circular	white	0.1	⊖	0.48x1.84	71.45	-	-	+	+	-	+	-	white
INEC*23	S	circular	clear	0.1	⊖	0.96x1.44	688.23	-	+	-	-	-	+	-	clear
VNEC*11	S	circular	white	0.08	⊖	0.96x2.95	9.00	-	+	-	-	-	+	+	white
VNEC*21	S	circular	clear	0.05	⊖	0.57x0.76	127.10	-	-	-	-	-	+	+	clear
Azotobacter sp.	S	circular	yellow	0.1	⊖	1.92x0.961	1157.30	-	+	-	+	-	+	-	yellow
Beijerenkia sp.	S	circular	white	0.1	⊖	1.057x0.96	2080.70	-	+	-	+	+	+	+	white
Azospirillum lipoferum CCM3863	M	circular	pink	0.1	⊖	1.44x0.961	76.20	-	-	+	-	-	+	-	pink
Azospirillum sp. UPMB10	M	circular	white	0.15	⊖	1.922x0.961	1287.60	-	-	+	-	-	+	-	white

Table 1. Continued

Isolate	Media	Morphology					ARA (nmol/mg/ day)	Biochemical assay							
		Colony			Gram staining			Voges Proskauer test	C-sources						PDA
		Form	Color	Size (cm)	Gram staing	Size of Cell (μm)			Mon	Mal	Rhm	Bas	Glu	Arb	
Azospirillum sp. UPMB13	M	circular	pink	0.05	⊖	1.44x0.961	2879.10	-	-	+	-	-	+	-	pink
Azospirillum brasilenese Sp7	M	circular	pink	0.01	⊖	0.961x0.864	1250.00	-	-	+	-	-	+	-	yellow
E.coli HB101	G	circular	yellow	0.2	⊖	1.20x2.00	-	-	+	-	+	-	+	+	yellow

Symbols : G, glucose media; S, sucrose media; M, malate media; CCM, combied c-source media +, strians positive; -, strains negative
Mon, Mannitol; Mal, Malate; Rhm, Rhamnose; Bas, Bensoate; Glu, Glucose; Arb, Arabinose.

APPENDIX B

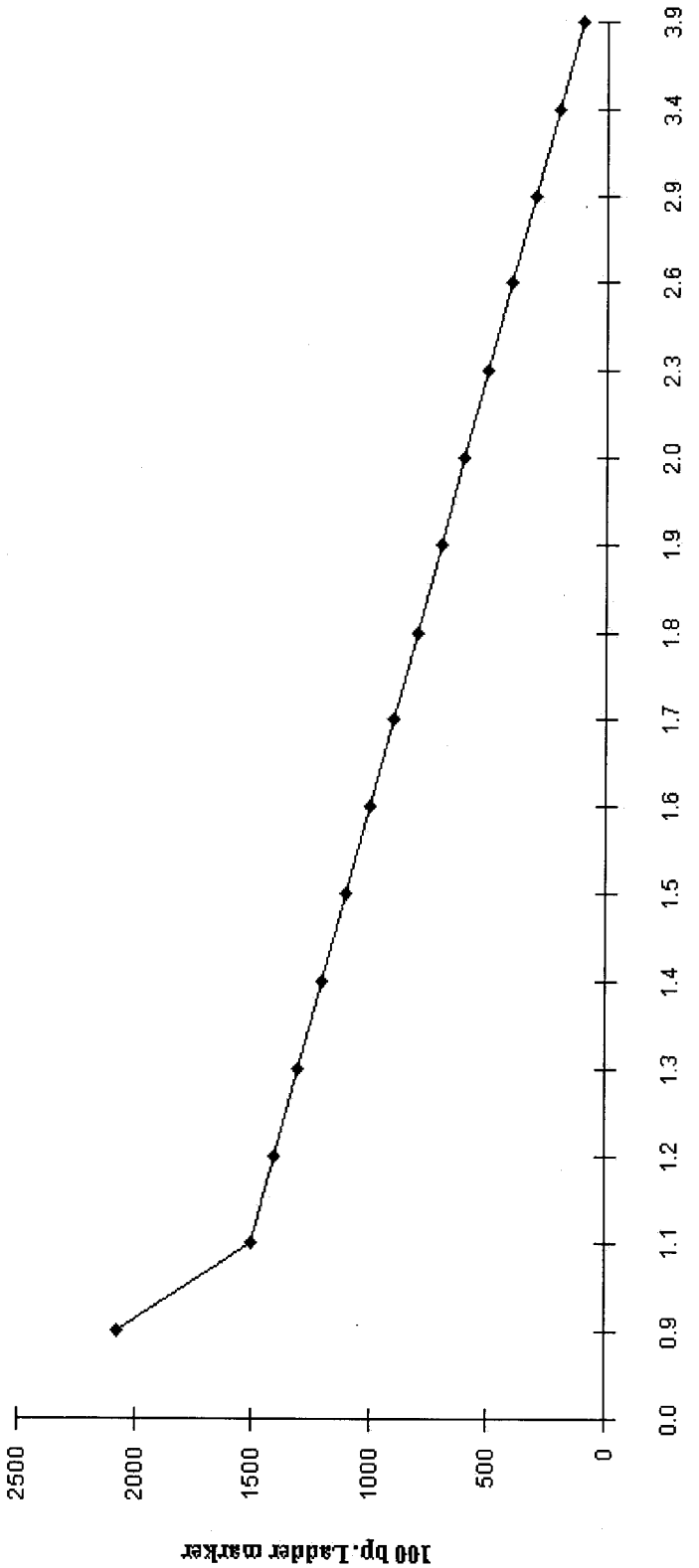

Figure 1. Standard curve of 100 bp. Ladder marker

BIBLIOGAPHY

Miss Orawan Piyaboon was born on 19 March, 1975 in Nakhonrachasima, Thailand. She graduated with the Bachelor degree of Science in Biology, Mahasarakham University in 1997. She presented research work in Proceeding : Research Reports on Biodiversity in Thailand, Biodiversity Research and Training Program (BRT), 12-15 October 1998, Khon-Khan, Proceeding : The 10th Annual Meeting of the Thai Society for Biotechnology and The 1998 Annual Meeting of the National Center for genetic Engineering and Biotechnology on Biotechnology for A Self-Sufficient Economy, 25-27 November 1998, Bangkok, Proceeding : Research Reports on Biodiversity in Thailand, Biodiversity Research and Training Program (BRT), 11-14 October 1999, Sungkha, Proceeding : The 5th Asia-Pacific Biochemical Engineering Conference 1999 and The 11th Annual Meeting of the Thai Society for Biotechnology, 15-18 November 1999, Phuket and Proceeding : Nitrogen Fixation Seminar Under Programme of Large Scale Cooperation (NRCT-JSPS/DOST/LIPI/VCC) 1-5 December 1999, Chiang Mai, Thailand.