Diversity of *Frankia* associated with *Alnus nepalensis* and *Casuarina equisetifolia* in West Bengal

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DECLARATION

I declare that the thesis entitled "Diversity of *Frankia* associated with *Alnus nepalensis* and *Casuarina equisetifolia* in West Bengal" has been prepared by me under the guidance of Dr. Arnab Sen, Associate Professor of Botany, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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This work is a tribute to my teachers

Late Sri Subodh Kumar Adhikari

Late Sri Arun Sarkar

Sri Sankar Deb Goswami

&

Sri Kumarendra Bhattacharya

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Dated:

Place:

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Abstract

Dutch microbiologist and botanist Martinus Willem Beijerinck (1851-1931) first discovered the process of biological nitrogen fixation. It is the ability to convert the molecular nitrogen into cellular nitrogen. This process is a unique property of a group of free living, associative and symbiotic prokaryotic microorganisms. During this nitrogen fixation process, an enzyme complex called nitrogenase complex catalyzes an ATP dependent reduction of atmospheric nitrogen to ammonium. This fixed nitrogen is the primary source of available nitrogen in nature. It is very difficult to calculate any direct correlation among nitrogen fixing bacteria, however the process of biological nitrogen fixation and its enzyme system is very similar in all nitrogen fixers. They are distributed among 27 families and 80 genera of Eubateria (including cyanobacteria) and three thermophilic genera of archaebacteria.

The bacterium *Frankia* is an important member of biological nitrogen fixer. This bacterium is a microaerophilic, gram positive to gram variable; sporulating actinomycetes belong to the Darmatophilous group. They produce characteristic sporangia and vesicle. This morphology differentiates *Frankia* from other actinomycetes. *Frankia* make symbiotic association for biological nitrogen fixation with a number of woody dicotyledonous collectively known as actinorhizal plants.

The present work deals with the diversity of this bacterium isolated from coastal region to the Darjeeling hills. For diversity study the bacteria was isolated and characterized. *Frankia* were isolated from the root nodules of *Casuarina equisetifolia* and *Alnus nepalensis* using a newly standardized technique. The isolated strains showed typical *Frankia* culture characteristics in liquid Q_{mod} as well as in nitrogen free defined propionate minimal medium (DPM). The vesicles were produced only in DPM medium. Visible nodule and typical root hair deformation was observed on *Casuarina* seedlings in reciprocal inoculation. However the seed germination of *Alnus* and its survival, in both *in vitro* and *in vivo*, is a serious challenge to scientists as the germination process is very slow. Mature seeds of *Alnus* were collected from the healthy plants of Darjeeling hills. Some of the surface sterilized seeds were overnight soaked in aerated water, three different media WPM, MS and Hoagland in half and full strength was used. For the study of effects of hormones in seed germination, the media were separately supplemented with 1-5mg/l NAA and IBA singly or in combinations. Maximum germination was observed in Hoagland media for both treated and untreated seeds, whereas minimum in ½ MS for treated and ½ WPM for untreated seeds. IBA and

NAA+IBA were found to be more effective than NAA in terms of germination percentage and rooting. Good rooting and its growth were maximum in the media supplemented with IBA(@ 3, 4 & 5mg/L) These experiments showed that germination of *A. nepalensis* seeds have specific hormonal requirements, which is fulfilled by mycorrhizal and associative fungal association.

Frankia strains isolated from both *C. equisetifolia* and *A. nepalensis* were subjected to various concentrations of heavy metal salts to study the heavy metal resistance pattern of *Frankia*. This experiment was a part of physiological characterization of *Frankia* of these regions. *Frankia* strains isolated from *C. equisetifolia* were studied in sensitivity to nickel, copper, lead, cadmium and cobalt salts. They were found to be resistant to high concentration of cadmium and cobalt salts. Heavy metal salt resistant patterns of *Frankia* isolated from the *A. nepalensis* were also prepared. The strains were highly resistant to cadmium chloride and lead nitrate, moderately resistant to cobalt chloride and less resistant to nickel chloride and copper sulphate. The heavy metal resistant *Frankia* colonies were re-isolated from various plates and preserved for further studies. A characteristic heavy metal induced either purple or red or both type of pigment production was noted in *Frankia* strains isolated from the *A. nepalensis* in all the cases except in lead at MIC or higher concentrations. This type of pigment production was totally different from the pigment production of the strains isolated from *C. equisetifolia* growing in North Bengal University campus where a faint red pigment was produced only in lead. It was found that *Frankia* isolated from *C. equisetifolia* could tolerate higher heavy metal salt concentration than *Frankia* strain isolated from *A. nepalensis.*

The availability of complete genome sequences of *Frankia* strains from different bio-geographic locations gives us an opportunity to analyze the codon usage of the heavy metal resistance genes, predict their expression level in comparison to protein coding genes and ribosomal protein genes. CLUSTAL W was employed to the nucleotide sequences of studied heavy metal résistance genes to find out the diversity. All the protein coding genes, ribosomal protein genes, and genes for heavy metal resistances were analyzed by the software CodonW (Ver. 1.4.2) and CAI calculator 2. The heavy metal resistance genes clustered along with the ribosomal protein genes and exhibit strong codon bias. The Nc/GC3 plot was done, which demonstrated an effective technique for investigating codon usage variations among the genes. Genes coding ribosomal proteins that are known to be highly expressed were highlighted in the NC/GC3 plots. Compared to the ribosomal protein genes

the heavy metal resistance genes in the genomes showed some difference. Especially the heavy metal resistance genes of CcI3 were less biased compared to the ACN14a and EAN1pec strains. Most of the ribosomal protein genes and heavy metal resistance gen for all the *Frankia* genomes were found to be clustered at lower ends of the plot suggesting a strong codon bias for these genes. Genes with effective number of codons value <40 had much stronger codon bias than be simply explained in terms of mutational bias. Ribosomal protein genes and those associated with metal resistance had a lower mean Nc value than that obtained for all of the protein coding genes suggesting a higher degree of bias in the former. These values indicated a strong codon bias over mutational bias. This codon bias was aroused due to natural selection for translational efficiency.

Codon Adaptation Index is a simple, effective measure of synonymous codon usage bias. The index uses a reference set of highly expressed genes from a species to assess the relative merits of each codon, and a score for a gene is calculated from the frequency of use of all codons in that gene. In ACN and EAN heavy metal genes and ribosomal protein genes had higher mean CAI values compared to that of the protein coding genes. On an average the CAI values of protein coding genes are high. High CAI values indicate better expression levels. Especially the comparatively higher expression levels for heavy metal resistance indicated the ability of *Frankia* genomes to survive in stressed environments and subsequent adaptability. The result obtained in this study put an additional support to the hypothesis that *Frankia* strain CcI3 is more symbiotic than saprophytic.

The heavy metal resistance genes of *Frankia* evolved in a single major clade and two subclades. Clustering of copper resistance and tellurite resistance genes of the different *Frankia* strains suggests that they have co-evolved as a unit. The Zn-Co-Cd resistance gene has a completely different origin and does not lie in a particular clade. Clustering of copper resistance and tellurite resistance genes of the different *Frankia* strains suggested that they had co-evolved as a unit. The putative Zn-Co-Cd resistance gene which is basically a cation diffusion facilitator family transporter had a completely different origin and did not lie in a particular clade. The study of heavy metal resistance has great significance since the heavy metal resistance is a more suitable marker for slow growing bacteria like *Frankia* as it is more stable than antibiotics resistance.

Eleven *C. equisetifolia* and *A. nepalensis* specific *Frankia* strains were isolated and characterized on the basis of organic acid decarboxilation, protease and β-glucosidase

activity as well as utilization of twelve different carbon sources. Three strains isolated from *C. equisetifolia* and two strains isolated from *A. nepalensis* showed decarboxylation and protease activity. All the strains utilize the Na-propionate, Na-acetate and Na-succinate as carbon source in nitrogen free medium in general. The strains isolated from NBU Campus could also utilize fructose and sucrose in addition. The result thus obtained was analyzed with the software POPGENE to calculate the diversity among the *Frankia* strains by Nei genetic diversity, Shannon index, single locus component and two locus component and Wahlund effect. *Frankia* population present in this area shows very low genetic diversity and the total genetic diversity was intrapopulation in origin and divided into two different physiological groups.

The entire *Alnus* specific strains along with CeSi10, CeSi11 and CeSi12 cluster in one group, and the rest of the *Casuarina* strains made other group. Among the strains all alder and CeSi10, CeSi11 and CeSi12 belong to physiological group A and CeSt2, CeSt5 CeSt9 of *Casuarina* strains belong to physiological group B. Field emission scanning electron microscopy was also performed and *Frankia* specific structures were found.

For the molecular biological work, DNA were isolated and purified both from culture and nodules. A new 16s r-DNA specific primers were developed and a 520 bp long portion of the said region was amplified. The amplicon was digested with *Alu*I, *Taq* I, *Hae* III, *Mbo*I and *Msp*I restriction enzymes. The PCR-RFLP profile was photographed. The results were analyzed with the software POPGENE to calculate the diversity among the *Frankia* strains by Nei genetic diversity and Shannon index. The results again showed that *Frankia* population present in this area had very low genetic diversity and the total genetic diversity was intrapopulation in origin. From the dendogram based on PCR-RFLP profile, a more or less distinguished pattern emerged in case of *Alnus* based *Frankia.* The *Casuarina* based *Frankia* were places in different clades. The probable reason of *Casuarina* based *Frankia* behaving differently from *Alnus* based *Frankia* could be that all the *Casuarina* plantations in West Bengal are exotic and were perhaps brought from different places.

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Chapter 1
INTRODUCTION

Dutch microbiologist and botanist Martinus Willem Beijerinck (1851-1931) first discovered the process of biological nitrogen fixation. While working in Wageningen University and Polytechnische Hogeschool Delft (Delft University of Technology), he thoroughly studied the various nitrogen fixing organisms and found that certain bacteria can convert atmospheric nitrogen into ammonia. He had isolated *Bacillus rudicicola* and proved that it forms nodules on the roots of members of the family Fabaceae. Later he isolated *Rhizobium* species, studied nitrogen fixation, and demonstrated nitrogen fixation by free-living microorganisms, particularly *Azotobacter chroococcum* (http:// en.wikipedia.org/wiki/ Martinus_Beijerinck). Some of his students also did excellent work in this field. Prof. A.H. Van Delden, who was a student of Prof. Beijerinck, worked in this field of microbiology. He worked on *Bacillus oligocarbophilus*, which is a nitrogen fixer. At the same time Sergei Nikolaievich Winogradsky (1856 – 1953) in his short working period in St. Petersburg (1891-1905) as chief of the division of general microbiology of the Institute of Experimental Medicine, first identified the ability of obligate anaerobe *Clostridium pastorianu* in fixing atmospheric nitrogen. Basically, in biological nitrogen fixation, the prokaryotic organisms convert the atmospheric nitrogen into ammonium ion, which is absorbed by the plants. *Frankia* is one of those prokaryotic organisms which have this unique property.

The bacterium *Frankia* is a microaerophilic, nitrogen fixing, gram positive to gram variable, sporulating actinomycetes belong to the Darmatophilous group. This bacterium has a high G+C content of 68-72% (An *et al*, 1983). It produces characteristic sporangia in submerged liquid culture and also form vesicles. This

morphology differentiates *Frankia* from other actinomycetes (Myrold, 1994). This bacterium produces three characteristic cell types, namely vegetative hyphae, sporangium and vesicle in the culture medium as well as *in planta* and it is assumed that these structures are also produced in the soil. In solid agar medium *Frankia* produce submerged colonies without aerobic hyphae. In liquid culture *Frankia* produce submerged growth with out any floating or aerial growth (Benson and Silvester, 1993). It is an obligate heterotrophic, microaerophilic, slow growing bacterium with a doubling time of about 15hr or more in culture; however their growth in symbiotic condition seems to be unrestricted, because timing of root infection, nodule development, and host cell infection are similar to that of rhizobia-legume system (Wall, 2000).

Vesicle is the site of the nitrogenase activity of *Frankia*. The lipid coating layers of the vesicle provide the necessary oxygen protection towards the nitrogenase. This property of the vesicle is revealed by Freeze fracturing technique of electron microscopy (Parsons *et al*, 1987). The actinorhizal nodules show an optimal nitrogenase activity at around 20K Pa of oxygen and significant inhibition above 25K Pa of oxygen is noted (Rosendahl and Huss.Danell, 1988; Silvester *et al*, 1990).

An (1985) was the first to use the DNA homology technique in classification of *Frankia* (An *et al.*, 1983; An *et al.*, 1985; An *et al.*, 1987). With the advent of Field emission scanning electron microscopy, Polymerase Chin Reaction (PCR) technology, Sequencing technology, DNA hybridization and various bioinformatics software like CodonW, CAI calculator etc gave a significant boost in the *Frankia* research. Various *Frankia* specific PCR primers targeting to 16s rRNA, 16s-23s rRNA spacer and *nif*D-*nifK* intergenic region have been developed. Workers used these techniques to study both pure cultures as well as uncultured *Frankia* directly from nodules (Benson *et al.*, 1996; Normand *et al.*, 1996; Murry *et al.*, 1997; Jamman *et al.*, 1993; Jeong *et al.*, 1999; Sur *et al.*, 2006, 2007, Bose *et al.*,2007; Ventura *et al.*,2007; Sen *et al.*, 2008, 2010). Thus, it has become easy to study the evolution, systematic and diversity of *Frankia* with the help of these molecular approaches. Recently the whole genome of six *Frankia* strains have been sequenced (e.g. *Frankia*

EAN1pec NC 009921; *Frankia* Eul1c NC 014666, *Frankia* NC 015657; *Frankia* ACN14a NC 008278; *Frankia* CcI3, NC 007777; and *Frankia* EUN1f, NZ ADGX00000000) (http:// www.ncbi.nlm.nih.gov/genome).

In addition to these molecular techniques, traditional culture based approaches are also on to elucidate the mechanism of nitrogen fixation by *Frankia*-actinorhizal system. Heavy metal resistance pattern (Richard *et al.*,2002 Bose and Sen.,2006, 2007) and antibiotic resistance pattern (Tisa *et al.*,1999) of *Frankia* have been established in the determine the effective genetic marker in *Frankia*.

Unlike *Rhizobium*, *Frankia* make symbiotic association with a number of woody dicotyledonous plants collectively known as actinorhizal plants (*action* from actinomycetes, *rhiza* the plant root) (Tjepkema and Torrey, 1979). To be precise *Frankia* infects about 194 species of dicotyledonous plants belonging to 25 genera, 8 families and 7 orders. Surprisingly there are representatives of actirhizal plants in almost every orders cutting across the evolutionary lineage (Benson and Silvester, 1993, Normand and Fernandez, 2006). The nitrogen fixing capability of those plants under the influence of *Frankia* helps them becoming key representative of different ecosystem and various agro forestry programme. These plants fixed sustainable amount of molecular nitrogen, which ranges from 240-350 kg N_2 $ha^{-1}y^{-1}$ and comparable with those of leguminous plants (Dawson, 1990; Hibbs and Cromack, 1990; Wheeler and Miller, 1990, Wall, 2000). This nitrogen improves the nitrogen condition of the soil and this nitrogen fixing capability is advantageous to the yield of associated plant group and reduces the use of chemical nitrogenous fertilizers. They increase the yields of associated crops by way of soil nitrogen with adding litters. They also increase the concentration of nutrients to the surface of the soil by extracting them from the deeper layer. Y. R. Dommergues (1987) postulated six important concepts for optimal utilization of biological nitrogen fixation in agro forestry which are as follows:

a. Plant species with high nitrogen fixing ability with good adaptability should be used.

b. Identify and multiplication of best host.

c. The best should show high nitrogen

fixing potential in field condition.

d. The host should provide with required nutrients for expression of full nitrogen fixing potential.

e. Secondary effects should not be overlooked

f. The host should be analyzed through short term and long term method.

Among the actinorhizal species *Alnus* and *Casuarina.* (Akkermans and Houvers, 1983; Gauthier *et al.,* 1984; Bond, 1983) are important for agro forestry.

Casuarina, a member of Casuarinaceae, consists of 82 species, mostly from Australia, South-East Asia and the Pacific islands. Johnson (1982) recognizes four genera under this family: *Casuarina* (e.g *C. equisetifolia, C. cunninghamiana, C. obesa, C. junghuhniana,* syn. *C. montana, C. Oligodon, C. glauca,*); *Allocasuarina* (e.g., *A. torulosa, A. littoralis, A. fraseriana, A. stricta, A. decaisneana*); *Gymnostoma* (e.g., *G. rumphiana, G. deplancheana, G. papuana*), and *Ceuthostoma. Casuarina* is suitable for agroforestry systems. One classical example is found in Papua New Guinea, where *C. oligodon* and *G. papuana* are used in the highlands and *C. equisetifolia* used in the lowlands, as intercrop with food crops, and are used as shade trees (Thiagalingam, 1983; Bourke, 1985). In India, *C. equisetifolia* plantations are associated with crops such as peanuts, sesame and various pulses (Kondas, 1981). *Casuarina* is planted as windbreaks. *C glauca* is used in this purpose in Tunisia and *C. equisetifolia* is used in Senegal. *Casuarina* has profuse nodulation property and this property of *Casuarina* explains its high nitrogen -fixing potential. A selected clone of *Casuarina equisetifolia* was reported to fix 42.5 g of N_2 per tree during the first nine months following plantation. *Casuarina* can tolerate high degree of salt concentrations. *C. obesa* can tolerate 15,000 ppm of chloride. So, they are considered as good candidates for the reclamation of salt-affected soils (Bajwa, 2004).

On the other hand, genus *Alnus* belongs to the family Betulaceae. Important *Alnus* species are *A nepalensis, A. viridis, A. glutinosa* etc. They are also good candidates for agro forestry. *Alnus* prefer cool and moist climate with mean annual temperature of 13-26°C and mature trees are frost tolerant. They can grow up to 3000m with annual rainfall of 500-2500mm. They can also grow on non fertile hill soil. *Alnus* is introduced in several

countries for species trial in agro forestry which include Bolivia, Uganda, Burundi and Java (Bajwa, 2004).

The improvement of soil texture and environmental condition by actinorhizal nitrogen fixation depend upon the improvement of host and symbiont, that is *Frankia.* A well characterized *Frankia* with good host can change the scenario of nitrogen deficient soil in the countries like India.

Therefore The Research Needs-Difficulties in *Frankia* Research

In comparison to other field, modern *Frankia* research has not grown up satisfactorily. The slow growth rate of *Frankia* and presence of contaminations are responsible behind this phenomenon (Benson and Schultz, 1990). The growth rate is the characteristic of an organism and a very little is left in the hand of workers. However researcher can reduce or eliminate the contamination. Sodium hypochlorite and osmium tetra-oxide are the potent candidates for this purpose (Benson and Silvester, 1993). Corrosive nature of osmium tetra-oxide creates problem in the isolation of *Frankia.*

Another difficulty in *Frankia* research is the absence of a universal media for isolation and maintenance of pure culture. *Frankia* is a poor competitor for carbon sources in comparison to its contaminants. The cortical layers of actinorhizal root nodules are heavily contaminated with soil microorganisms. The growth rate of which is generally faster than the *Frankia.* So, the prerequisite for successful isolation of *Frankia* was to eliminate these contaminants without damaging the endophyte in the cortical tissue. Lechevalier and Lechevalier (1990) suggested simple tap water agar, define propionate minimal medium to complex Qmod (Lalonde and Calvert, 1979) medium for isolation and maintenance. Bose and Sen (2006) pointed out the problems in using the two types of media. The minimal medium suppressed the contaminations, which may grow upon transfer into richer medium and on the other hand richer medium invite contaminations from environment. *Frankia* also need various nutritional supplements for growth (Lechevalier *et al.,* 1982). The isolation problem also inhibits the sequencing based diversity work. Limited work is done in recent years and six strains of *Frankia* genome have been sequenced (e.g.

Frankia EAN1pec NC 009921; *Frankia* Eul1c NC 014666, *Frankia* NC 015657; *Frankia* ACN14a NC 008278; *Frankia* CcI3, NC 007777; and *Frankia* EUN1f, NZ ADGX00000000) (http:// www.ncbi.nlm.nih.gov/genome)

The criteria used in classifying *Frankia* include the morphology, chemistry, plant infectivity, serology and DNA homology (Lechevalier, 1984). Although in order to differentiate strains a few physiological tests have been developed (Lechevalier *et al.*, 1983; Horriere, 1984; Weber *et al.*, 1988). But limited work had done in this field of microbiology. The molecular biological work is also very limited in this field. Overall very limited work has been done in Indian *Frankia* and particularly in *Frankia* associated with *C. equisetifolia*. A few work has been done in the *Alnus* associated *Frankia* (Sen, 1996; Bajwa, 2004). So, there are mammoth work is left in the field of *Frankia* research in India. In short the difficulties are summarized under the following heads:

- After almost thirty years of its isolation in pure culture, the isolation of *Frankia* in pure culture still remains a very difficult job to do. Development of a suitable easy technique for isolation of *Frankia* in pure culture is one of the prime necessities of *Frankia* research.
- Physiological and morphological characterization of *Frankia* strains have not been done widely and in India this kind of characterization work is almost absent. Thus physiological and morphological characterization of *Frankia* is another important field of *Frankia* research which need immediate attention.
- The genetic diversity of *Frankia* is that kind of field where a considerable work has been done, but satisfactory result needs more rigorous work in this field with new primers and statistical methods.

Thus The Objective of The Present Study

- Survey and collection of germplasms from various parts of Darjeeling hills for *Alnus* based *Frankia* and different regions of coastal Bengal for *Casuarina* based *Frankia*.
- Isolation of *Frankia* in pure culture.
- Morphological characterization of pure culture of *Frankia* with field emission scanning electron microscopy.
- Biochemical characterization of

isolated *Frankia* found in hilly and coastal Bengal.

- Preparation of heavy metal stress resistance profile of *Frankia* found in hilly and coastal Bengal.
- Study of GC composition, codon usage profile, codon preferences and codon adaptation index of heavy metal resistance genes of *Frankia* of both *Alnus* and *Casuarina* based *Frankia* strains found in public domain along with *Elaeagnus* strain.
- Isolation of genomic DNA directly from nodules and strains.
- PCR amplification of parts of 16s rRNA gene of *Frankia* with newly designed primer and restriction digestion of the amplicon.
- Determination of diversity among cultured and uncultured *Frankia* strains from hilly and coastal Bengal from the data generated.

Chapter 2
REVIEW OF LITERATURE

2.1 Relevance of Biological Nitrogen Fixation Research

All the living organisms required nitrogen for their daily needs like preparation of proteins, enzymes etc. It is a triple bonded, stable, almost inert colourless, odorless gas. In Earth's atmosphere there is about 77% of nitrogen which is conserved through nitrogen cycle (Rosswall, 1981). The cycle includes various steps which includes nitrogen assimilation, mineralization, nitrification, denitrification and biological nitrogen fixation. Most plants and microbes preferably assimilate the ammonium salts as a source of nitrogen through either glutamate dehydrogenase (GDS) or glutamine synthetase/ glutamate synthase (GS/GOGAT) cycle. This GS/ GOGAT cycle is widely and preferably used by organisms (Miflin and Lea, 1977). The ability to convert the molecular nitrogen into cellular nitrogen is called biological nitrogen fixation. This is an unique property of a group of free living, associative and symbiotic prokaryotic microorganisms. This fixed nitrogen is the primary source of available nitrogen in the nature.

In the modern technological era, scientists invented another industrial method, called Haber-Bosch process to produce nitrogenous fertilizer as an artificial source of nitrogen. This process is very energy consuming and complicated. However it is very demanding as the demands for nitrogenous fertilizer increases day by day as nitrogen (N) is the most commonly used limiting nutrient used for plant growth. Ladha and Reddy (1995) estimated the average energy used in ammonia production in Haber-Bosch process. It is well known that

the efficiency of the industrial process varies considerably, but it is estimated that about 1.3 ton of oil (or equivalent amount of energy) is required to fix 1 ton of NH_3. The annual production of about $77x10^6$ ton of NH_3- N in the year 1996 requires about 0.1 x 10^9 ton oil or equivalent. This is about 1.4% of the total fossil fuel consumption in the year 1995. (Patyk and Rheihardt, 1997; Scholz *et al.*, 1997). The combustion of such oil or coal add a huge amount of pollutant to the environment. Unfortunately our chemically produced nitrogenous fertilizer dependent green revolution can not afford any minimization of such figure which may result in food shortages due to inability of the industry to produce nitrogenous fertilizers.

After the Second World War, colonial powers lost their control over major parts of the globe and either democratic or socialist governments came to the power almost throughout the world. The main challenge of these responsible governments was to fight against hunger and poverty and feed their population that is to give the minimum food security to them. In the 20^{th} century grain crop yields have constantly increased and reached a remarkable level in many areas of agriculture throughout the world. In India it is generally known as "Green revolution". This green revolution occurred due to introduction of genetically improved cultivars of crop plants as well as use of chemical pesticides and of course use of huge amount of nitrogen fertilizers. These cultivars are very nitrogen-responsive varieties and produce large reproductive structures which can accommodate more grain. This is the reason for increase of use of total nitrogenous fertilizer between 1950 and 1990. The increase was about 10 fold. Recent studies show that the yields of the major cereal crops and utilization of nitrogenous fertilizer did not increase in the last decade. In some instances like Japan, United States of America, Western Europe it decreased slightly. On the other hand, farmers in Asian countries like India and China uses huge quantity of nitrogen fertilizer. It is estimated that there is a massive gap between the nutrient inputs from external sources and the constant drain of nutrients from the soil due to crop removals and soil erosion. In India, the estimated nutrient deficit was about 10 million tons in 1990, and is likely to touch new summit with more intensive cropping systems and

increasing soil degradation and finally leading to loss of soil fertility (Bumb and Baanante, 1996).

If the fertilizer demands of next few years are considered, it is found that the food needs will increase and it will come from a nearly static land base. Thus, a more intensive agriculture with more and more use of fertilizer is expected. Bumb and Baanante (1996) based on an econometric model estimated 1.2% increase in the world fertilizer use from 1990 to 2020. It is estimated that the expected fertilizer use will rise from 143.6 million tons to 208 million tons from 1990 to 2020. It is very interesting to note here that the rate of increase of nitrogen fertilizer is much lower than that compared with the period of 1960-1990 (Buckley, 2000). Bumb and Baanante (1996) explained this lower rate as 'a higher base, limited potential for further growth and changing policy environment. It is because of the already high application rates, environmental concerns, reduction in farm support programme and trade liberalization'. Buckley (2000) supported the calculation of Bumb and Baanante (1996) and estimated an annual 1.1% increase in nitrogenous fertilizer use from 1990 to 2070.

So, it is established that nitrogen fertilizer reached its peak and left very limited potential for growth in the present century and it is the time to search an alternate source of fertilizer which would be environment friendly and have higher potential for growth. In a global scenario of reaching nitrogen resource to a plateau, rising concerns over possible environmental effects of chemical fertilizers, as well as their cost for small-scale farmers in developing countries, it is essential to expand the use of the biological nitrogen fixation (BNF) technologies that offer the greatest environmental and economic benefits for each specific agro-ecosystem.

Biological nitrogen fixation is generally found in prokaryotic system. *Eriphorum vaginatum*, an eukaryotic plant, is the only exception (Chapin *et al.*, 1993). It is very difficult to calculate any direct correlation among nitrogen fixing bacteria, however the process of biological nitrogen fixation and its enzyme system is very similar in all nitrogen fixers. They are distributed among 27 families and 80 genera of eubateria (including cyanobacteria) and 3 thermophilic genera of archaebacteria (Elkan, 1992). This process has four universal

requirements, which are as follows:

1. An enzyme system, called nitrogenase enzyme complex.

2. A high energy requirement and availability (ATP).

3. Anaerobic condition for nitrogenase activity.

4. A strong reductant.

During the nitrogen fixation process nitrogenase complex catalyzes the ATP dependent reduction of atmospheric nitrogen to ammonium and is composed of two protein called dinitrogenase and dinitrogenase reductase. Dinitrogenase is the most important enzyme of the system, which reduces the atmospheric dinitrogen into ammonia at appropriate temperature and pressure (Peters and Szilagyi, 2006).The dinitrogenase protein is a $\alpha 2\beta 2$ tetramer of 240 KDa that are encoded by the gene *nif*KD. The complex also contains two types of metal centres, the P clusters (8Fe-8S), which bridges the α and β subunits and an unique iron –molybdenum co-factor (FeMo-Co), which is buried within the α subunit and is the site of substrate reduction (Dean *et al*, 1993). The α and β subunits of nitrogenase is linked with each other by cysteine residues of molybdo-ferro protein (Rubio and Ludden, 2008). Dinitrogenase reductase is a 60KDa homodimer that is encoded by the gene *nif*H. It specifically reduces dinitrogenase, apparently transferring elements to the P-cluster, which then channels them to FeMo-Co metalocluster. In addition to this catalytic role, dinitrogenase reductase is also involved in the biosynthesis of FeMo-Co and the maturation of dinirtogenase (Filler *et al.*, 1986).

The biological nitrogen fixing organisms can be grouped into three categories- freeliving, associative and symbiotic. Free living diazotrophs was first described by Winogradrodsky. They can fix sustainable amount of nitrogen either by any specialized structure like heterospore of cyanobacteria or in anaerobic or partial anaerobic condition in nature. The symbiotic organisms form an association with a particular host plant and fix nitrogen with in a specialized structure called nodule. Associative nitrogen fixing bacteria are newly invented group of micro organisms. They are the predominant microbial flora of root rhizosphere of particular host plant, but do not enter into the host or host do not produce any specialized structure for them (Elkan,

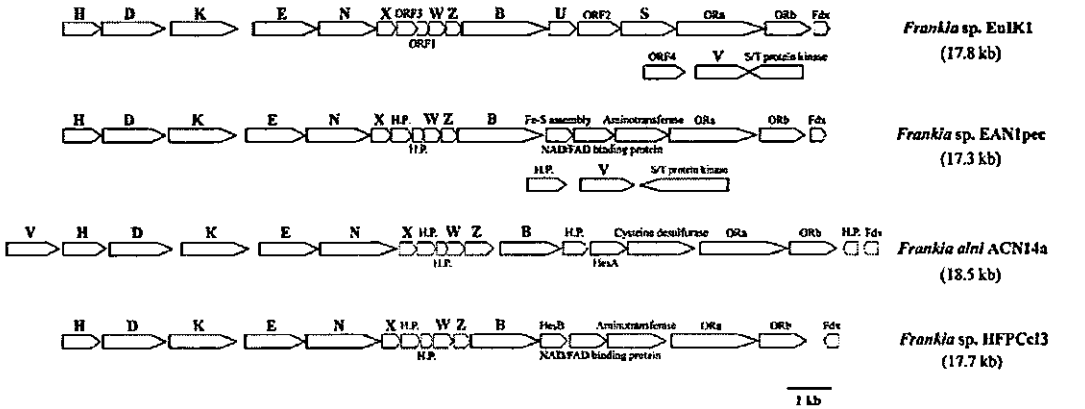


Fig 2.1 Comparison of the physical organization of *nif* and *nif*-associated genes from EulK1 strain with those from the 3 full-genome sequenced *Frankia* strains. Gene organization in the *nif* clusters are highly conserved among *Frankia* strains, except with regards to *nif*V location. In *Alnus* infecting strains, *nif*V was located just upstream of *nif*H, whereas the gene was found in a different genomic location in *Casuarina* and *Elaeagnus* infecting strains, including EuIK1 strains. Bold letters represents the nif gebnes, H.P = Hypothetical protein, Fe-S assembly accessory proteins (in EAN 1pec strains)
(Adopted from: "Organization of nif gene cluster in *Frankia* sp. EuIK1 strain, a symbiont of Elaeagnus umbellate' Chang Jae Oh • Ho Bang Kim • Jitae Kim •Won Jin Kim • Hyoungseok Lee • Chung Sun. An Arch Microbiol (2012) 194:29–34DOI 10.1007/s00203-011-0732-7)

1992).

2.1.1. Bacterial Genes Responsible For Biological Nitrogen Fixation

Primarily three gene families are associated with the process of biological nitrogen fixation. They are *nif, nod* and *fix* gene families. The *nif* genes are responsible for the production of nitrogenase (*nif*D and *nif*K) and dinitrogenase reductase (*nif*H) enzymes. Several *nif* genes are identified and are sequenced from various groups of microorganisms, which include *nif*E, *nif*N, *nif*V, *nif*B, *nif*S, *nif*A etc. They are structurally conserved (Fischer, 1994). The *fix* genes are those genes which have a definite role in nitrogen fixation and found in rhizobia, but their homologous counterpart in *Klebsiella pneumoniae* is not found. They are first identified in the symbiotic organism *Rhizobium meliloti* (Corbiun *et al.*, 1983; Earl *et al.*, 1987; Ruvkun *et al.*, 1982). Their presence was noted in several other rhizobia latter on, which includes *Bradyrhizobium japonicum,* (Fuhrmann *et al.*, 1985; Gubler and Hennecke, 1986) *R. leguminosarum bv. trifolii,* (Iismaa *et al.*, 1989) etc. Unlike the *nif* genes the *fix* genes are situated on a single operon and associated electron transport to nitrogenase. Important *fix* genes are *fix*A, *fixB*, *fix*H, *fix*N, *fix*O, *fix*X, *fix*R etc (Fischer, 1994). The *nod* genes are associated with the process of nodulation in legume- rhizobia system. *Frankia* genome contains some putative nod like genes, however, true *nod* gene is

Table 2.1 List of Actinorhizal plants ((Benson DR and Silvester WB, 1993; Sen 1996).

Plant Order	Plant family	Host plant genus
Fagales	Betulaceae	*Alnus*
Protales	Elaeagnaceae	*Elaeagnus, Hippophae, Shepherdia*
Rhamnales	Rhamnaceae	*Ceanothus, Colletia, Kentrothamnus, Retanilla, Telguenea, Trevoa, Discaria,*
Myricales	Myricaceae	*Myrica, Comptonia**
Casuarinales	Casuarinaceae	*Casuarina, Allocasuarina, Gymnostoma, Ceuthostoma*
Ranunculales	Coriariaceae	*Coriaria*
Rosales	Rosaceae	*Drayas, Purshia, Cercocapus, Cowania, Chamaebatia*
Violales	Datiscaceae	*Datisca*

* *Frankia* was first isolated from this plant

Table 2.2: Classification of nodulated plants of Rosid I linages (Wall. 2000).

Nitrogen fixing plant lineage	Nitrogen fixing plant Families
Lineage I	Myricaceae, Casuarinaceae and Betulaceae
Lineage II	Elaeagnaceae, Rhamnaceae and Rosaceae as well as *Parasponia* (Ulmaceae member, being infected by *Bradyrhizobium*)
Lineage III	Coriariaceae and Datiscaceae
Lineage IV	Fabaceae (Includes non actinorhizal legume plants)

absent in its genome (Franche *et al.*, 2009). The *nif* genes of *Frankia* are highly conserved. The size varies from 17.3kb (*Frankia* sp. EulK1) to 18.5 kb (*Frankia* strain ACN14a) (Chang *et al.*, 2012). The physical organization of *nif* and *nif*-associated proteins of various *Frankia* genomes are shown in Fig 2.1.

2.2 The Actinorhizal Plants

A group of dicotyledonous plants that are nodulated by *Frankia* is called actinorhizal plants. They are distributed among eight orders, eight families, 24 genera and 194 species of land plant (Table 2.1) (Benson and Silvester, 1993, Normand and Fernandez, 2006). These plants are found in different hemisphere of the earth ranging from arctic Tundra (*Dryas* spp.) and alpine forest (*Alnus* sp., *Coriaria sp.* etc) to costal and xeric condition (*Casuarina sp.*)(Fig 2.2). *Frankia* in symbiosis with actinorhizal plants fixed sustainable amount of molecular nitrogen, which ranges from

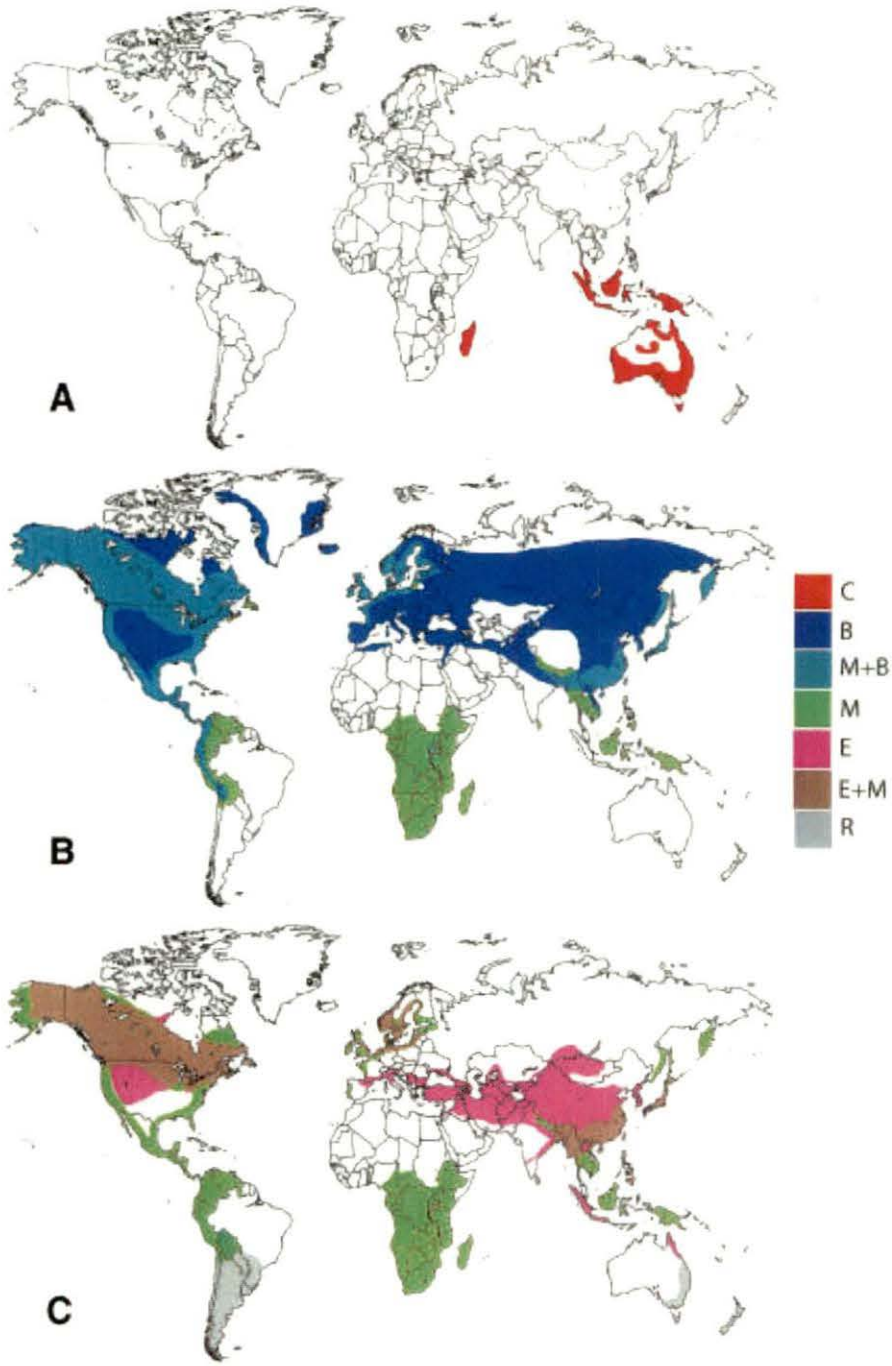


Fig 2.2 Present-day native distribution of actinorhizal plant hosts. (*A*) Distribution of plant hosts for CcI3, including *Casuarina* and *Allocasuarina* of the Casuarinaceae (C). (*B*) Distribution of plant hosts for ACN, including *Alnus* sp. in the Betulaceae (B) and Myricaceae (M) and their overlap (M+B). (*C*) Distribution of plant hosts for EAN including members of the Elaeagnaceae (E), Myricaceae (M), and the actinorhizal Tribe Colletieae of the Rhamnaceae in South America, Australia, and New Zealand (R). Elaeagnaceae and Myriceae (E+M) overlap in some areas. Maps were drawn with information from Silvester (1977) and from the Missouri Botanical Garden Web site (www.mobot.org). (Adopted from 'Genome characteristics of facultatively symbiotic *Frankia* sp. strains reflect host range and host plant biogeography' Philippe Normand *et al*, 2007 Genome Res. 2007 January; 17(1): 7–15.)

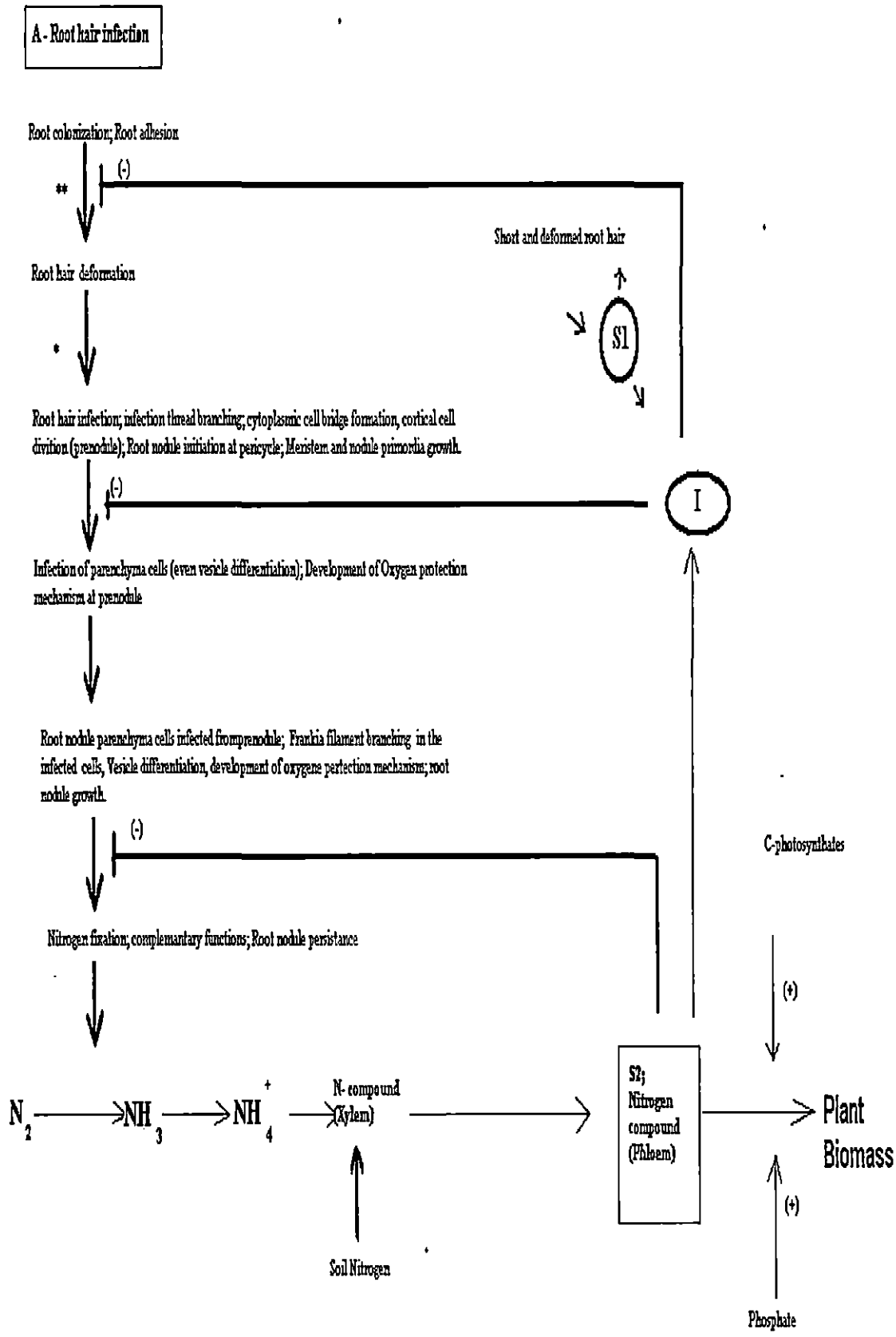


Fig 2.3 Model A for regulation of nodulation in actinorhizal symbiosis in root hair infection in *Frankia*. Thin lines means transient pathway. * = Second symbiotic interaction and molecular recognition step involving flavonoids. ** = First molecular signal involving the root hair deformation factor. (-) = Inhibition, (+) = Activation. S = Signal molecule, I = Inhibitor. (Wall, 2000)

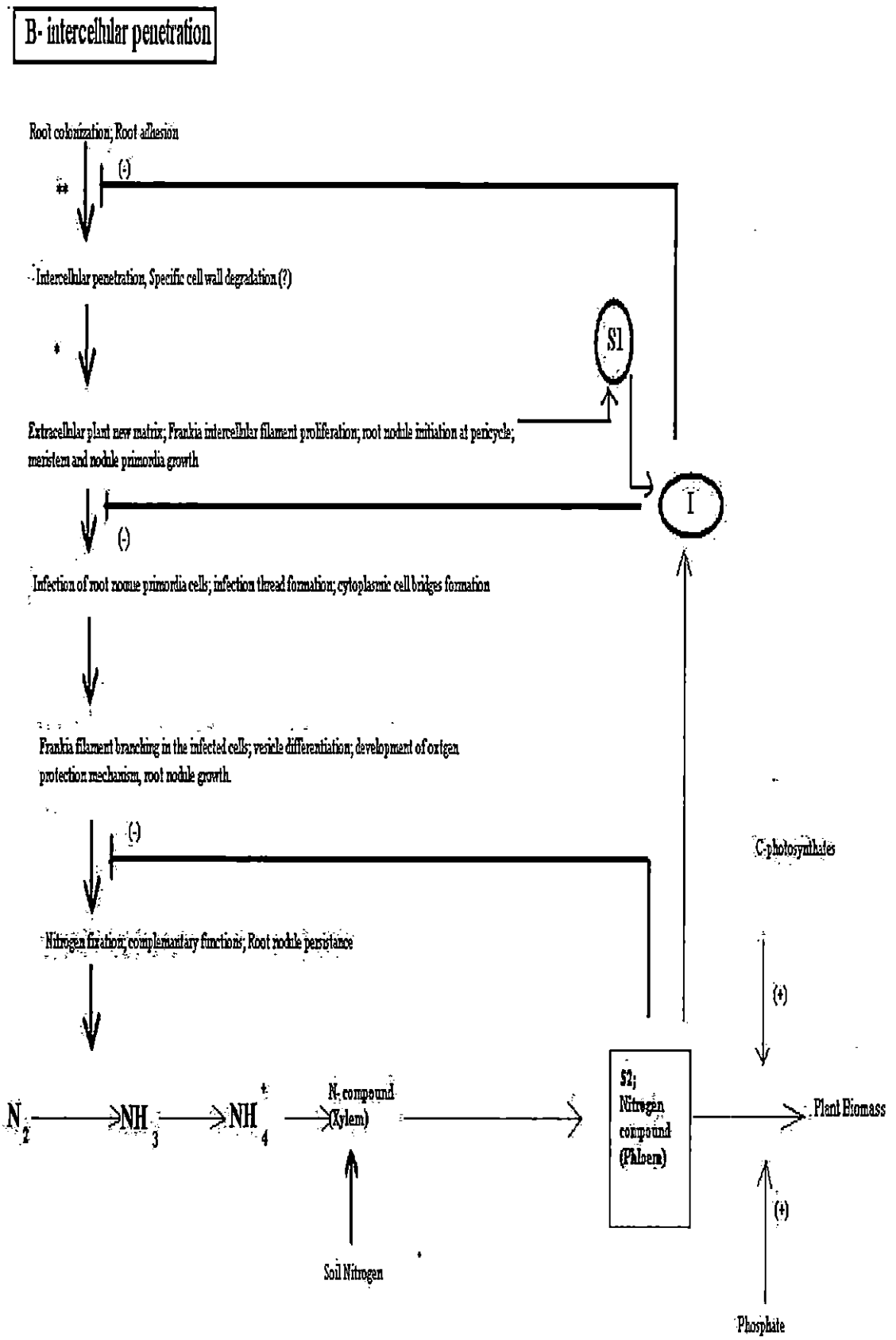


Fig 2.4 Model B for regulation of nodulation in actinorhizal symbiosis intercellular penetration of *Frankia*. Thin lines means transient pathway. * = Second symbiotic interaction and molecular recognition step involving flavonoids. ** = First molecular signal involving the root hair deformation factor. (-) = Inhibition, (+) = Activation. S = Signal molecule, I = Inhibitor. . (Wall, 2000)

240-350 kg N_2 $ha^{-1}y^{-1}$, which is comparable to those of leguminous plants (Dawson, 1990; Hibbs and Cromack, 1990; Wheeler and Miller, 1990; Wall, 2000). Since actinorhizal plants are the early visitors of marginal soils, they are considered as pioneer species in the landslides and other threatened areas. These plants are also used as wood, fiber, food, chemicals, etc (Benoit and Berry, 1990; Diem and Dommergues, 1988, 1990; Hibbs and Cromack, 1990; Myrold, 1994). In India, research on actinorhizal plants is limited. An excellent work has been done by Basistha *et al.* (2010) on *Hippophae salicifolia* growing in Lachen and Lachung valleys of North Sikkim where snowfall, heavy rain and landslides are very common. They found that this actinorhizal plant is growing as dominant species in different ecological and topological condition in that region. Recently, Bargali (2011) studied habit, habitat, distribution and possible ecological significance of eight actinorhizal plants found in Kumaun Himalayan region.

Three major phylogenetic subgroups of actinorhizal plants have been identified (Swensen, 1996). The first subgroup, includes symbiotic taxa from the families Betulaceae, Myricaceae and Casuarinaceae. The second subgroup, includes symbiotic taxa from the families Datiscaceae and Coriariaceae. The third subgroup, includes symbiotic taxa from the families Rhamnaceae, Rosaceae and Elaeagnaceae (Tab 2.2) (Swensen, 1996). The nodules formed by members of different actinorhizal plants differ considerably regarding organization of infected cells in the cortex (i.e. symbiotic, nitrogen-fixing cells), oxygen protection mechanisms for nitrogenase, patterns of *Frankia* differentiation, infection mechanisms, and organization of carbon and nitrogen metabolism (Pawlowski and Bisseling, 1996; Berry *et al.*, 2004, 2011; Schubert *et al.*, 2010, 2011).

The actinorhizal root nodules are established through a series of interactions between the host actinorhizal plant and the bacterium, *Frankia.* The actinorhizal root nodules are perennial with coralloid structure. It consists of multiple lobes. The *Myrica* and *Ceanothus* lobes are discrete and in *Alnus*, they are densely packed. Each nodule is a modified lateral root. The actinorhizal roots contain vascular cylinder, cortical tissue and periderm. The cortical cells get infected with the *Frankia.* From the apex of the mature

root nodule negatively geotrophic nodule root is developed. This nodule root does not take part in the nitrogen fixation process (Bajwa, 2004).

The ideal condition for root infection and subsequent nodule development is not well understood in *Frankia*-Actinorhizal system. *Frankia* hyphae were embedded in the mucilage layer of the root hair. It is established that a single infection is enough for nodule development. However, in the natural condition more than one infection occur in the nodule formation. In laboratory conditions the frequency of nodulation is directly proportional to the amount of inocula (Newcomb and Wood, 1987). It is interesting to note that root hairs of actinorhizal plants are deformed by several non-symbiotic soil bacteria (Knowlton *et al.*, 1980). These soil bacteria play an important role in the *Frankia*-actinorhizal interaction. They are called 'Helper' organisms. *Pseudomonas sp.* helps in the nodule formation in *Alnus* and *Casuarina* in controlled condition (Knowlton *et al.*, 1980).

The bacterium follows two different pathways for infection of the host tissue. It takes place either by root hair deformation as found in *Alnus, Casuarina, Myrica* etc (Callaham *et al.*, 1978, 1979; Berry and Torry, 1983) or intracellularly as in the case of *Ceanothus, Elaeagnus,* etc. Some strains can follow both the pathways for infection (Miller and Baker, 1986).

The signal molecule of these plants bear same basic chemical feature. Actinorhizal plants undergo feedback regulation of nitrogen fixing symbiosis with *Frankia,* which involves at least two different and consecutive signal molecules that leads to a mechanism controlling root nodulation in actinorhizal plants (Wall, 2000).

The mechanism of infection in actinorhizal plants follows two different ways –

i) root hair infection (*Casuarina sp.*) and

ii) intercellular infection (*Shepherdia sp.*).(Fig 2.3 and 2.4)

Frankia has very little or no host specificity as found in case of other nitrogen fixing bacterium like *Rhizobium.* In rhizobia–legume symbioses *nod* signal molecules helps in the nodulation and form the basis of host specificity (Oldroyd *et al.*, 2009) These kind of *nod* factors are absent in *Frankia.* The bioassay of reporter gene is also absent in *Frankia* (Céremonie *et al.*, 1999). On the basis of the study of

actinorhizal root hair deformation (Gherbi *et al.*, 2008; Markmann *et al.*, 2008) and signal factors of arbuscular mycorrhizal fungi (Maillet *et al.*, 2011), it is predicted that *Frankia nod* factor equivalents are a chitin-based molecule. Recently a LysM-type mycorhizal receptor was found in the infection process of *Rhizobium* symbiosis of the nonlegume *Parasponia andersonii* (Op den Camp *et al.*, 2011). This finding suggested that *Frankia* might have developed a unique pathway of synthesis of a novel chitin-based signal molecule unrelated to *Rhizobium* which functions in a *nod* factor-independent infection mechanism (Pawlowski *et al.*, 2011). In nodule, *Frankia* followed a unique pathway for nitrogen utilization. Unlike rhizobial symbiosis, the primary nitrogen assimilation might take place in *Frankia* and stored as arginine (Berry *et al.*, 2011).

Frankia is also able to produce a special structure called vesicle to protect its nitrogenase from O_2. Exception is *Casuarina* nodules, which provide a partially anoxic environment for *Frankia* to fix nitrogen by producing oxygen diffusion barrier by class II hemoglobin (Berg and McDowell, 1987, 1988; Jacobsen-Lyon *et al.*, 1995). However *Casuarina* infecting *Frankia* strains are capable enough to produce vesicle. In fact *Casuarina* - *Frankia* does produce vesicle to protect their nitrogenase from the toxic effect of oxygen, when growing in pure culture (Sen, 1996). *Frankia* strains have been shown to synthesize truncated hemoglobins for protection of nitrogenase from oxygen (Tjepkema *et al.*, 2002; Beckwith *et al.*, 2002; Coats *et al.*, 2009). Pawlowski *et al.* (2007) showed that in *Datisca glomerata* nodules, the plant also produces truncated hemoglobin in infected cells. It is involved in nitric oxide (NO) detoxification, not in O_2 transport as class I hemoglobin. The presence of truncated hemoglobin in the *D. glomerata* cells infected with *Frankia*, and the presence of large amounts of a class I hemoglobin in alder (Sasakura *et al.*, 2006) and *Myrica gale* nodules (Heckmann *et al.*, 2006) indicates that, as in legume nodules (Horchani *et al.*, 2011), large amounts of nitric oxide are produced in actinorhizal nodules, leading to high levels of stress. Class II haemoglobins are generally believed to contribute to oxidative stress in nodules by producing reactive oxygen species (ROS) such as superoxide anions (O^{2-})

and hydrogen peroxide (H_2O_2) (Becana *et al.*, 2000). This has been confirmed by the fact that RNAi inhibition of *leghemoglobin* gene transcription in nodules of the legume *Lotus japonicus*. It not only increase free O_2 and the loss of nitrogenase and nitrogen fixation in nodules, but also reduces H_2O_2 contents (Günther *et al.*, 2007). For *C. glauca*, it has been suggested that CgMT1, a class I type 1 metallothionein protein, could be part of the antioxidant system to prevent ROS accumulation in the nitrogen-fixing cells of the nodule (Obertello *et al.*, 2007). Additionally, Tavares *et al.* (2007) have shown that *Frankia* contributes to ROS production in nodules. Thus, both legume and actinorhizal nodules have to cope up with high levels of stress. Altogether, it should be pointed out that actinorhizal plants have a greater ability to tolerate extreme environmental conditions. Progress on research on growth condition of actinorhizal plants indicates that they grow in relatively adverse conditions than legumes, and therefore, it is also possible that they possess enhanced antioxidant based defense systems (Pawlowski *et al.*, 2011). Goyal *et al.* (2011) studied various extracts of *H. salicifolia* and found that it possess high antioxidant activities, which is helpful in preventing or slowing the progress of oxidative stress.

From the above discussion it is apparent, that there are two different ways of mechanism with two different sets of receptor and signal molecules. The intercellular infection takes place at a much faster rate than that of root hair infection. A flavonoid compound is involved in this process (Hughes *et al.*, 1999). However, the identity of the compound is not yet clear. There are several environmental and bacterial factors involved in nodulation. The environmental factors involve light, water, availability of nitrogen and phosphate, soil pH, pCO_2 and pO_2. The bacterial factors are concentration of the inocula, physiological state of the strain and nitrogen fixing ability of the bacterium. These factors play important role in the function, regulation and development of the nodules in actinorhizal plants (Fig 2.5). From the work of Valverde and Wall (1999), it is found that the tap root system consists of a temporary window of susceptibility for nodulation. Wall (2000) proposed two different models for the regulation of nodulation in actinorhizal plants which involved two

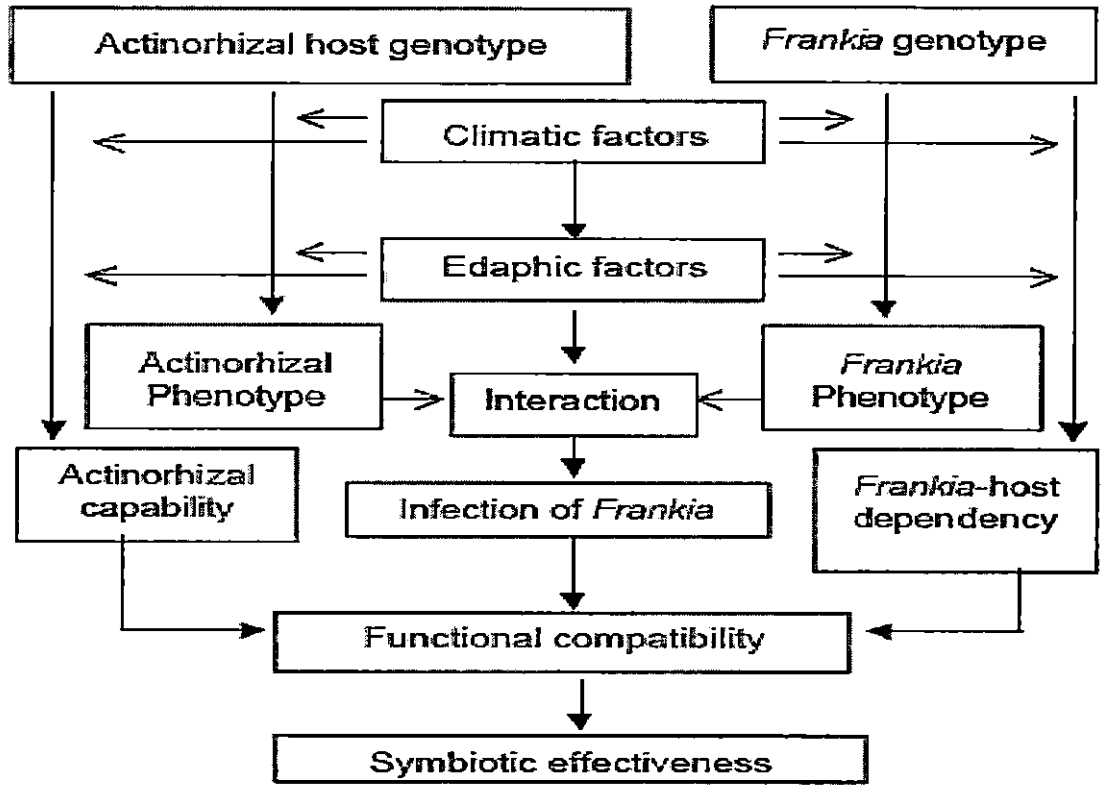


Fig 2.5 Complexity of host–*Frankia* interaction and symbiotic. (Adopted from "*Frankia*–actinorhizal symbiosis with special reference to host–microsymbiont relationship." By Verghese and Misra, 2002)

unknown signal molecule (S1, S2) and an unknown inhibitor molecule (I). These are two-step models and the signal and inhibitor molecules regulate the whole process by three independent pathways as follows:-

- Opening and closing of susceptibility window for interaction.
- Arresting nodule primordia at stages before host cell invasion by *Frankia* and vascular bundle differentiation.
- Inhibiting nodule development in the growing root (Fig 2.3 & 2.4).

Normand and his co-workers had proved the existence of the bacterium *Frankia* in more than 100 Million years ago on the basis of *nifH* gene sequence (Normand and Bousquet, 1989). The actinorhizal symbiosis may evolve almost in similar time (Sen, 1996). There are two different hypotheses that have been projected regarding the evolution of the actinorhizal symbiosis. According the first hypothesis, widely related plant genera acquired the capacity of making symbiotic relationship with *Frankia* to achieve selective advantages in certain

ecological niches. So, acquisition of the symbiotic character had hardly taken place before the divergence of families into their respective genera. The second hypothesis stated that the available soil nitrogen was limited in the early Cretaceous period (Bond, 1983) and a group of plant was forced to make association with nitrogen fixing soil bacteria *Frankia* to survive in struggle for existence with other wind pollinated angiosperms largely belonging to Magnoliidae. Some of these plants later evolved into the actinorhizal plants. During the last 100M years the availability of nitrogen in the soil increased due to nitrogen fixation and some of the ancient plants lost their symbiotic nitrogen fixing habit. Although this ancient property remains as a selective advantage in some pioneer plants which are now called actinorhizal plants. The DNA hybridization study by Bousquet *et al.* (1989) on host plants support the second hypothesis.

The *rbcL* gene sequence shows that the actinorhizal nitrogen fixing plants are grouped in Rosid I linages of the seed plants (Fig 2.6). The property of nitrogen fixation in nodules may evolve in a single time in the earth's history (Doyle, 1998). This Rosid I group may be subgrouped into four linages, of which three are actinorhizal and the fourth one is Fabaceae (Soltis *et al.,* 1995). The fossil records, geographical distribution, morphological and anatomical studies extended supports to this hypothesis (Wall, 2000). However, on the basis of 16s r-DNA sequence, *Frankia* can be divided into three clades and one '*Frankia* like clade'. The *Frankia* strains have been successfully isolated from Clade I and Clade II; however there is no isolates from Clade III *Frankia*. The existence of this clade is proved by PCR amplification of the 16s-rDNA. The analysis of data shows that the *Frankia*-Actinorhizal symbiosis follows a polyphyletic line of evolution and this occur at least three or four times in the history of evolution which clearly rejects the idea of Doyle (1998). This new idea was supported by Swenson (1996), Benson and Clawson (2000) and Jeong *et al* (2000). There are a few *Frankia rrn* gene sequences available for the study. From these sequences the evolutionary distances can be measured. This type of study was made in *Frankia* by Normand *et al.* (1996), Jeong *et al.* (1999) and Clawson *et al.* (2004) with different conclusions. The Ochman's

metric (Ochman and Wilson, 1987) is recognized as the best estimate for this type of work (Normand and Fernandez, 2006) and according to this metric, ancestor of *Frankia* evolved at about 350 MY ago from a group of soil actinomycetes. During this period the first trace of land plant was found. This ancestors of *Frankia* underwent a second radiation about 100MY ago giving birth of *Frankia*. During this period the first dicotyledonous plant families started to appear in the earth. This estimation that is very close to the fact that *Frankia* clusters emerged at 100-200MY ago, which corresponds well to the appearance of the oldest actinorhizal plant genera like *Myrica* and *Alnus* (Normand and Fernandez, 2006).

Compared to rhizobia, it is found that *Frankia* has relaxed host specificity. *Frankia* has very broad host range and restricted in clade-to-clade interaction. The plants belong to Hammamelidae clade is nodulated by the *Frankia* strains belong to clade-I. Elaeagnaceae and Rhamnaceae clade is nodulated by clade II *Frankia* and clade III *Frankia* nodulate the Rosaceae plants. So in conclusion, it can be said that this specificity largely resides on a group of signal molecules or a group of molecule that have same chemical backbone as well as common characters. The primitive actinorhizal plants, namely *Myrica* and *Gymnostoma* produce nodules when infected by both Clade I and Clade II *Frankia*. This is a notable exception and this observation established primitiveness of these plants and on this basis they can also be considered as the most primitive actinorhizae. A probable explanation of this infection lies in the habitat of *Gymnostoma* and *Casuarina*. *Casuarina* is native to drier Australian continent while *Gymnostoma* diversified in the wetter Melanesia region from the tertiary era beginning 65 MY ago.

2.3. The *Frankia*

The root nodules of non-leguminous plants invite attentions of researchers from early nineteenth century. Meyen (1829) first speculate on the nature of the cause of nodule and stated that it is a result of parasitic infection in the root. Woronin (1866) studied the detailed anatomy of the nodules and found some round vesicle and hyphae like structure within it. He found that the hyphae were passing through the intracellular region. He also noticed that there are some hyphae with round vesicular swelling tips. Woronin

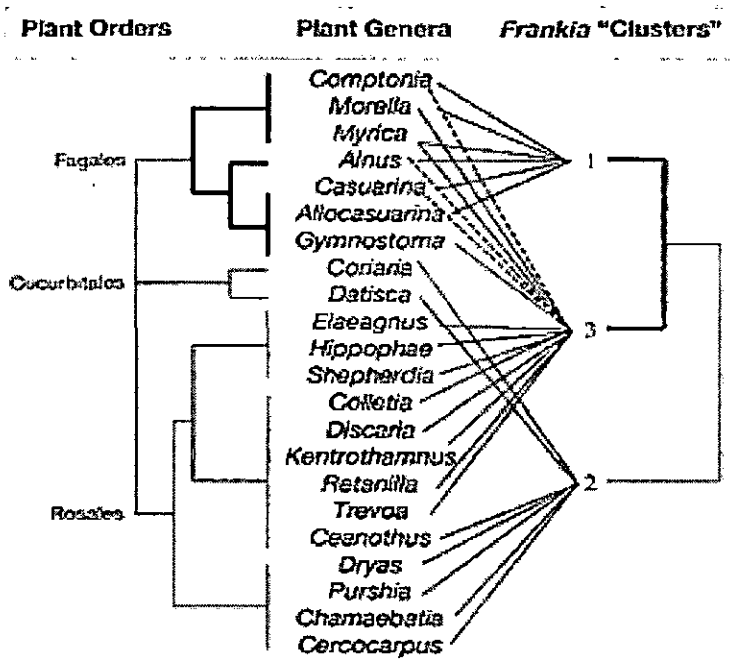


Figure 2.6. Correspondence between the plant genera RbcL (left) and *Frankia* 16S rRNA (right) phylogenies. Heavy lines - infection via root hair. Lighter lines - root penetration. ((Clawson *et al.*, 2004);web.uconn.edu/mcbstaff/benson/Frankia/PhylogenyFrankia.htm). The oblique lines join the plant and bacterial taxa when they can establish symbiotic structures, dashed lines indicate laboratory only symbioses not found in nature. (photo curtsey: Prof. Phillippe Normand, University of Lyon1, France).

considered the endophyte as a fungus and coined the name *Schinzia alni* as this unknown organism showed resemblance with a fungal parasite called *Schinzia cellulicolia* (Sen, 1996). Brunchorst, (1886) studied the cytological difference of leguminous and non-leguminous roots and named the endophyte as *Frankia subtilis* to honor his teacher eminent Swiss microbiologist A.B. Frank (1885), who, ironically did not believe in the presence of living microorganism in any kind of nodules and considered the structures as protein granules. Later Frank changed his idea on the nature of endoiphyte and along with Brunchorst considers *Frankia* as a fungus. On the other hand, the name *Frankia alni* was also coined by Von Tubeuf (1895) as a tribute to A.B. Frank. Several synonyms of *Frankia* have been proposed later on, which includes *Plasmodiophora alni* (Woronin) (Möller 1885), *Frankiella alni* (Woronin) (Maire and Tison 1909), *Aktinomyces alni* (Peklo 1910), *Actinomyces alni* (von Plotho 1941), *Nocardia alni* (Waksman 1941), *Proactinomyces alni* (Krassil'nikov,

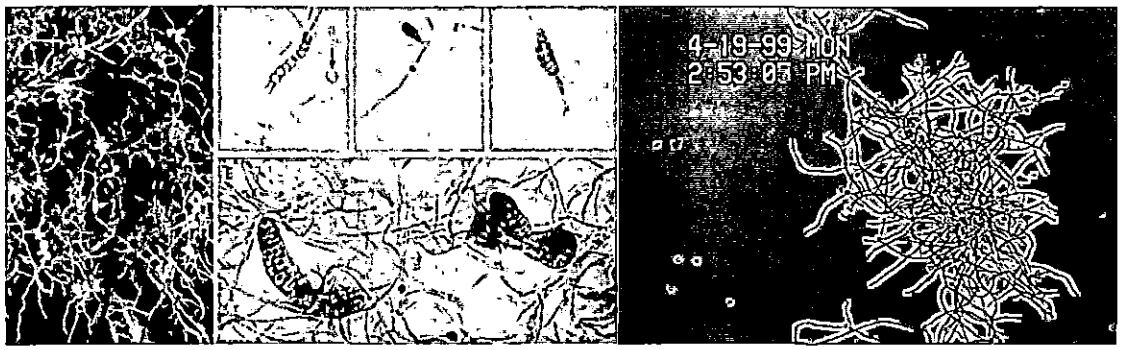
1949, 1959), *Streptomyces alni* (Fiuczek, 1959) (Normand & Fernandez, 2006).

Hiltner (1898) for the first time identified the endophyte as member of actinomycetes which is a close alley of *Streptomyces* while studying the roots of *Alnus* and *Elaeagnus*. (http:// www.mcdb.ucla.edu/Research/Hirsch/ images b / HistoryDiscoveryN2fixingOrganisms.pdf; Quispel, 1990). Hellriegel and Wilfarth published two papers during 1886-1888 on the fixation of atmospheric nitrogen by leguminous nodules with the help of the bacteria residing in the cortical layer. In their paper they introduced two terminology 'nitrogen user' and 'nitrogen accumulator' and showed differences between them. They identified Alders as nitrogen accumulator, and undoubtedly established source of nitrogen for growth of plants. Their work opened up a new avenue of research in plant microbial science (Bottomley, 1912; Quispel, 1988; Sen, 1996).

Hiltner in his later work with Alders demonstrated that young Alders can not survive in nitrogen free soils without root nodules. This result pointed out that though Alders are nitrogen accumulator they are certainly not the nitrogen fixer on the soil and another organism must be involved in the process of nitrogen fixation.

In another land mark study, Beijerinck isolated bacteria from legume root nodules which failed to infect non leguminous nitrogen accumulator and other plants proved that leguminous microsymbionts and non-leguminous microsymbionts are two different organism. (Brewin, 2002; Pawlowski and Bisseling,1996). Finally Krebber identified non-leguminous microsymbiont as actinomycetes in 1932 (Quispel, 1990).

Pommer (1956) was probably the first worker who was successful to isolate a slow-growing actinobacteria from nodules of *Alnus glutinosa* (Fig:2.7) which had unique morphological features like hyphae, multilocular sporangia and vesicles in pure culture. About 0.6mm colonies of that bacterium were obtained after 2-3 weeks of growth in glucose-asparagin agar described by Waksman for actinomycetes (1950). Unfortunately this strain was lost before independent studies in different laboratories. In the year 1964, electron microscopic study of root nodules of *Alnus glutinosa* and *Myrica cerifera* reestablished that these



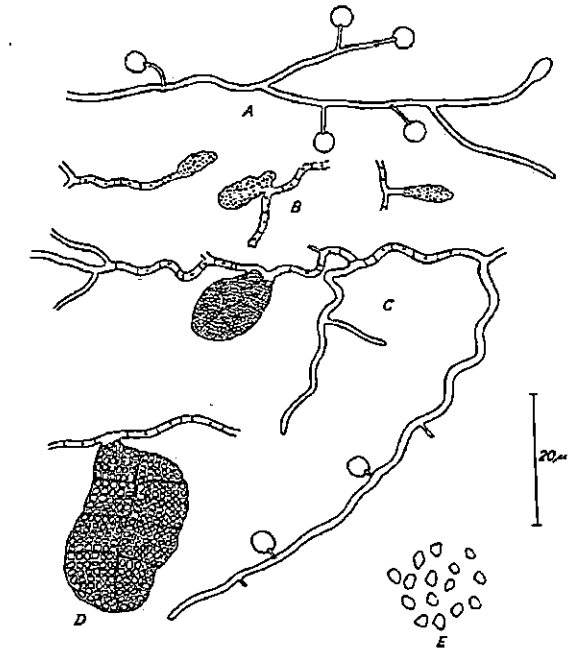


Fig 2.7 Some of the early photographs of Frankia. (photos provided by Prof. Phillippe Normand, University of Lyon1, France and Prof Louis S Tisa, University of New Hampshire, USA

Abb. 1. Der Wurzelknöllchen-Endophyt von *Alnus glutinosa* auf Glukose-Asparagin-Agar. Erläuterungen im Text.

root nodules were inhabited by actinomycetes bacteria as the electron microscope found prokaryotic structure in them (Becking *et al.*, 1964; Silver, 1964). From then efforts were on to isolate this unknown prokaryotic endophyte in pure culture. At last Callaham *et al* (1978) first isolated *Frankia* from *Comptonia peregrina* in pure culture followed by other workers (Diem and Dommergues, 1983; Diem *et al.,* 1983 and Sarma *et al.,* 1998). Lalonde (1978) worked on *Frankia* strain CpI1 isolated by *Callaham et al* (1978) and proved its ability to reinfect the host plant and established its symbiotic nature. The morphological nature of the bacterium isolated by *Callaham et al* (1978) shows unexpected resemblance with the lost strain of Pommer which he isolated from *Alnus glutinosa* nodules in 1959 (Benson and Silvester, 1993).

Because of slow growth of *Frankia* and frequent contamination by other fast growing organisms, fungi and other

actinomycetes are often mistaken as *Frankia*. To overcome this problem Lechevalier and Lechevalier, (1984) proposed following definition of *Frankia*:

"Actinomycetic, nitrogen fixing, nodule forming endophytes or endoparasite that have grown in pure culture *in vitro* and that:

a. induce effective or ineffective nodules in a host plant and may be re-isolated from within the nodules of that plant, and

b. produce sporangia containing non-motile spores in submerged liquid culture, and may also form vesicles.

c. free living actinomycetes having no known nodule forming or nitrogen fixing capacity, but that show the morphology described above."

Frankia produces three different kinds of cellular structure during its growth in pure culture or in symbiotic condition i.e. vegetative hyphae, multilocular sporangia containing spores and vesicle (Myrold, 1994, Benson and Silvester, 1993; and Akkermans and Hirsch, 1997). However, *Casuarina* infecting *Frankia* does not produce vesicle *in planta* (Sen, 1996).

2.3.1 Parts of Frankia

A. Hyphae

The actively growing cell type of *Frankia* is hyphae (0.5-2μ diameter) which form a mycelial mat by repeated branching. The vesicles and sporangia are produced from hyphae by differentiation. Aerial hyphae are absent on solid medium. The structure of *Frankia* hyphae, both *in vitro* and in symbiosis, was extensively reviewed by Newcomb and Wood (1987). Free-living organisms under the light microscope show branched hyphae. Under phase contrast and dark-field microscopes a large number of bright areas are seen within it (Benson and Silvester, 1993). They have type III cell wall and type I phospholipid content (Lechevalier *et al.*, 1982). Cell wall in chemically fixed materials appears to be composed of electron dense base layer (Lalonde, 1979; Baker *et al.*, 1980; Horriere *et al.*, 1983; Lancelle *et al.*, 1985). Cross walls originate from the base layer. A membranous layer some time may be visualized outside the outer wall layer (Newcomb *et al.*, 1979; Horriere *et al.*, 1983; Newcomb and Wood, 1987) and the cell wall, that may also contain spherical or

ovoid inclusions which can be visualized in both chemically fixed and freeze-substituted materials (Lancelle *et al.*, 1985). Internally hyphae cells contain numerous rosette-shaped glycogen granules (Benson and Eveleigh, 1979; Lancelle *et al.*, 1985) and lipid droplets. Individual ribosomes and polyribosomes are seen in freeze-substituted hyphal cells cytoplasm as relatively large (300nm) globular bodies. A large number of cytoplasmic tubules are present at the periphery of the freeze-substituted hyphal cell cytoplasm. They are circular in cross section (45 nm in diameter). These structures underlie the cell membrane both at cell septum and at the outside wall (Benson and Silvester, 1993; Lancelle *et al.*, 1985). An extracellular multilayered envelope was first identified in hyphae of free-living *Frankia alni* HFPCpII (Newcomb *et al.*, 1979). This structure is also present in vesicle of *Frankia* and this multilayered envelope has been identified in symbiotic hyphae of *Frankia* (Lalonde *et al.*, 1976; Berg and McDowell, 1987; Abeysekera *et al.*, 1990). This indicates that lipid enveloped hyphae may be common among the *Frankia* (Benson and Silvester, 1993).

B. Vesicles

Vesicles are the most definitive and characteristic structure of *Frankia* which is a unique developmental structure designed for physiological compartmentation and is totally absent in any other prokaryotic group (Benson and Silvester, 1993). In pure culture, vesicles are spherical, thick-walled and short stalked structure (2-6μ diameter) and *in planta*, they have variable shapes and their shapes are determined by their host. The multilaminate thick wall of the vesicle acts as a barrier to oxygen and protects the nitrogenase enzyme from toxic effect of oxygen (Parson *et al.*, 1987; Silvester *et al.*, 1990). These vesicles are the site of nitrogen fixation in free-living and symbiotic *Frankia* (Tisa and Ensign, 1987; Huss-Danell and Bergmann, 1990). The vesicles show a characteristic metabolism (Tisa and Ensign, 1988; Tisa, 1998). Although spores form the main mode of propagation, the vesicles can also give rise to vegetative hyphae (Schultz and Benson, 1989). They are lipid encapsulated, born either terminally or laterally to hyphae by a short encapsulated stalk and generally produced in nitrogen deficient medium

(Benson and Silvester, 1993).

Vesicles developed in nitrogen free medium first as a terminal swelling of hyphae or on short side branches. These early structures are separated by a septum near the base and are termed provesicle, which rapidly develop in to mature vesicles (2-4 μ in diameter) (Fontaine *et al.*, 1984). Provesicles are spherical cells, with dense cytoplasm (1.5-2μ in diameter), and they may show the initiation of internal compartmentation. The provesicles are unable to convert molecular nitrogen into ammonia nitrogen (Fontaine *et al.*, 1984). However some workers have reported the formation of vesicle in presence of available nitrogen in the medium (Gauthier *et al.*, 1981; Meesters *et al.*, 1987). Vesicles are observed as a bright structure under phase contrast microscope (Fontaine *et al.*, 1984) and also shows birefringence under polarized light microscope (Torrey and Callaham,1982), and can be seen as a bright halo under dark field microscope (Parsons *et al.*, 1987). The vesicle envelope and internal septations are the necessary prerequisite for nitrogenase activity and identified as two important and characteristic structural elements of mature vesicle in *Frankia* cells growing in culture, but neither of them appears essential in symbiosis (Benson and Silvester, 1993).

C. Sporangia and spores

Frankia strains are readily recognized by production of multilocular sporangia located either terminally or in an intercalary position (Newcomb *et al.*, 1979) on the hyphae in liquid culture. This morphology differentiates *Frankia* from other actinomycetes (Myrold, 1994). Sporangia (10 x 30-40 μ) contain multiple ovoid spores and are generally produced in stationary phase of growth (Myrold, 1994). They are also formed inside nodules of some actinorhizal plants (Schwintzer, 1990)

The *Frankia* sporangia were first identified in the nodules of *Alnus glutinosa* (Van Dijk and Merkus, 1976). They remain infective in dry soil for a long period (Tortosa and Cusato, 1991). On the basis of presence or absence of sporangia within a root nodule, *Frankia* strains have been classified as either spore$^+$ or spore$^-$ (Schwintzer, 1990) (Fig 2.8). Spore$^+$ strains appear to be much more infective than spore$^-$ strains (Normand and Lalonde, 1982), both spore$^+$ or spore$^-$ strains have been characterized at the molecular level (Simonet *et al.*, 1994). Filaments,

vesicles and sporangia have the infection potential; however the spores are the best material to infect. They are the major means for *Frankia* propagation in nature (Wall, 2000).

Chemically fixed developing spores show an electron translucent nucleoid region with dispersed fibrils, like the hyphae residue of a laminate envelope and numerous lipid droplets (Newcomb *et al.*, 1979). However the nucloid region is not evident in freeze-substituted preparation. The cytoplasm of the developing sporangia dispersed like that of the hyphae. The tubules are absent in the developed spores (Newcomb *et al.*, 1979). The sporangia developed by hyphal thickening followed by segmentation by septa originating from the inner layer of a double layered sporangial cell wall (Horriere *et al.*, 1983). This type of sporogenesis has been termed as enterothalic sporogenesis (Locci and Sharples, 1984). The immature spores are densely packed and are arranged in the middle portion of the sporangium, where as the mature spores are arranged in the peripheral region of the sporangia (Benson and Silvester, 1993). Mature spores are nonmotile, spherical or ovoid and measure about 1 -5μ in diameter (Benson and Silvester, 1993) (Fig:2.8).

2.3.2 Growth and physiology of *Frankia*

Frankia grow as filamentous colony on agar plates and they are generally grown in liquid culture In static batch culture the bacterium grow as submerged colonies with out any floating or aerial growth. They are obligate heterotrophic microaerophilic bacterium. *Frankia* is a slow growing bacterium with a doubling time of about 15hr or more in culture (Benson and Silvester, 1993); however their growth in symbiotic condition is seems to be unrestricted, since timing of root infection, nodule development, and host cell infection are similar to that of rhizobia-legume system. Thus difficulties to grow the bacterium in culture or isolating the bacterium are due to lack of knowledge in their growth conditions and growth requirements (Wall, 2000).

Some *Frankia* strains may produce red, yellow, orange, pink brown, greenish and black pigments in the medium. However, their pigment production depends on the strain, medium used and the age of the culture (Lechevalier and Lechevalier, 1984). A common red pigment has been identified as 2-methy

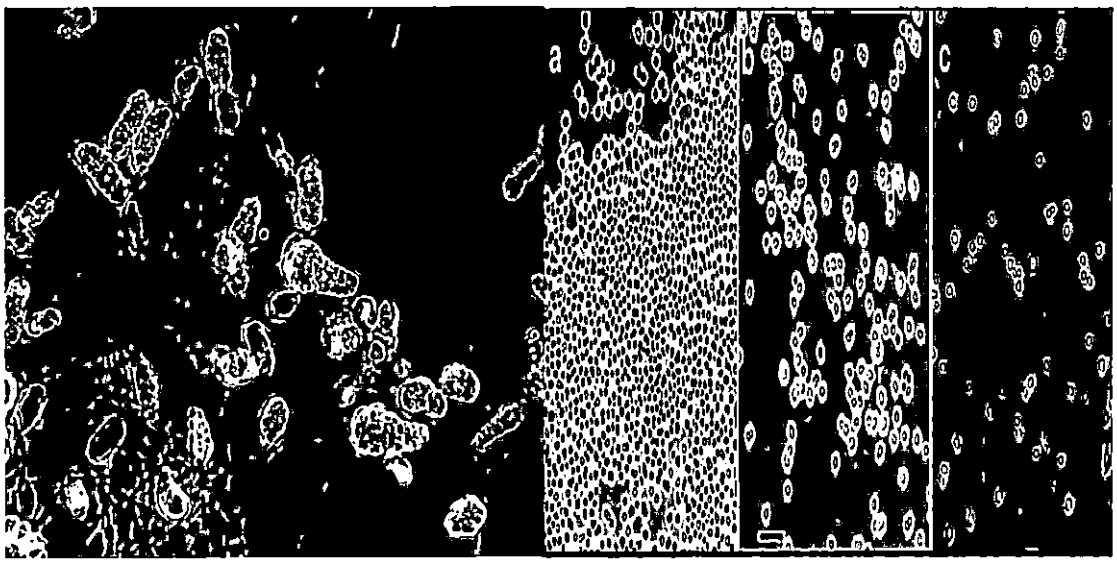


Fig 2.8 Micrograph of *Frankia* spores and sporangia. (Photographs are kindly provided by Prof Louis S Tisa, University of New Hampshire, USA)

1-4, 7, 9, 12, tetrahydroxy-5, 6-dihydrobenzo [a] naphthacene-8, 13-dione, a carboxylated derivative and a characteristic sugar 2-*O*-methyl-D-mannose (Mort *et al.*, 1983) have been identified in *Frankia* (Gerber and Lechevalier, 1984). Since pigmentation is highly variable, it is a poor criterion for placement in to genus but may also prove useful as a phenotypic trait for species recognition (Benson and Silvester, 1993).

Lechevalier divided the *Frankia* in two physiological groups (Lechevalier *et al.*, 1983; Lechevalier and Ruan, 1984); namely Gr-A and Gr-B. The Gr-A *Frankia* is more saprophytic, more aerobic and can be maintained in the slants. They show comparative rapid growth and diversity on serological and genetical basis. They also show protease and amylase activity and utilize various carbon sources which include arabinose, glucose, maltose, sucrose, trehalose, xylose, acetate, propionate, pyruvate and succinate. The Gr-B *Frankia* are more symbiotic and physiologically less active.

2.3.3 Heavy Metal Resistance of *Frankia*

Heavy metals are essential trace elements as they act as a cofactor of many enzymes and essential for growth in plants and microbes, but generally they are toxic at higher concentrations to all forms of life (Silver and Phung, 1996). The heavy metal resistance pattern arose much earlier than evolution of human beings on earth. This system arose soon after the life began in the world already polluted by volcanic activities and other geographical sources (Silver and

Phung, 1996). As this system has evolved over a long time so it attained a good diversity. (Bose *et al.*, 2007). These heavy metal resistance systems are important survival strategy for the bacteria. Bacterial chromosomes and plasmids contain genes for resistance to metal ions. They are frequently generating strong reactive oxygen species (ROS) and directly or indirectly causing gene mutations or protein damage. (Spain and Alm, 2003). More than fifty elements are available in the periodic table, which are designated as heavy metals. But the microbiologists are interested to only 17 of them. This is because rests are either not available or available in trace to the biological system. Weast (1984) estimated that elements at an average concentration lower than 1 nM are very un-likely to have any useful or toxic effect in biological system, and a resistance gene will not evolved for these metals. Tri- or tetravalent cations have very low solubility in water and because of the low solubility they have no biological significance. The remaining 17 heavy metals are important for study and grouped into three subgroups. The first subgroup contains Fe, Mo and Mn, which are important trace elements with low toxicity. The second subgroup contains Zn, Ni, Cu, V, Co, W and Cr, which are generally toxic but acts also as trace elements. Third subgroup contain well known toxic Ag, Sb, Cd, Hg, Pb and U ions which have little or no trace elemental activity (Nies, 1999).

The importance of study of heavy metal resistance pattern is three fold, firstly they acts as a genetic marker, secondly in bio-mining of expensive metals and thirdly heavy-metal-resistant bacteria may be used for bioremeditation of heavy metal contaminated environments (Nice, 1999).

The mechanisms of heavy metal resistance are generally resides in efflux pumping and enzymatic detoxification of metal ions. Occasionally, bio-accumulation or sequestration is a mechanism of resistance. Efflux pumps are the major currently known group of resistance systems with both plasmid and chromosomal systems. They can be either ATPase (e.g., the Cadmium and Copper ATPase of gram positive and Arsenite ATPase of plasmids of gram negative bacteria) or chemiosmotic (e.g., the divalent cation efflux systems of soil Alcaligenes and the Arsenite efflux system of the chromosome of

the gram negative bacteria and of plasmid of gram positive bacteria) (Silver and Phung, 1996).

In *Frankia* research the use of heavy metal resistance is very limited and focused on into genetic marker study only as there is a paucity of genetic marker in *Frankia*. In order to determine a genetic marker in *Frankia* system beside heavy metal resistance pattern (Richards *et al.*, 2002, Bose and Sen , 2006, 2007) and antibiotic resistance pattern (Tisa *et al.*, 1999) of *Frankia* have also been determined. *Frankia* strain ACN1^{AG}, Cc1.17, CcI3, CN3, CpI1-P, CpI1-S, DC12, EAN1pec, EI5c, EuI1c, EUN1f, and QA3 were studied for heavy metal resistance pattern. It is found that most of the *Frankia* strains are resistant to elevated levels of several heavy metal ions. The heavy metal resistance pattern is proved to be more effective than antibiotic resistance pattern due to the non degradable nature of heavy metal ions for long time of incubation (Richards *et al.*, 2002).

2.3.4 Taxonomy and Diversity of *Frankia*

Becking (1970) was for the first time tried to classify the family Frankiaceae with 10 species of unisolated symbionts in genus *Frankia* (Tab 2.3). His tentative classification was based on *in planta* morphology; vesicles morphology and he also considered this bacterium as an obligate symbiont. However many subsequent workers challenged Becking's view. After the successful isolation of *Frankia*, a good number of isolates were available for classical taxonomical approaches for classifying this bacterium. Lechevalier (Lechevalier and Lechevalier, 1984; Lechevalier and Ruan, 1984) proposed several taxonomic criteria for classification of *Frankia* which were based on ecology, infectivity, morphology, cell chemistry, physiology, serology, DNA homology and 16S rRNA profile. He proposed two *Frankia* types, "A" and "B". The type "A" correspond to Elaeagnaceae-infective (i.e. cluster 3) *Frankia* strains and the type "B" corresponds to *Alnus*-infective (cluster 1) strains. However this classification scheme did not meet satisfaction. Baker (1987) proposed a classification scheme (Fig 2.9) based on host infectivity which is as follows:-

- *Alnus* and *Myrica* infective group,
- *Casuarina* and *Myrica*, infective group
- Elaeagnaceae and *Myrica* infective group and
- Those infective only on *Elaeagnus*

Table 2.3. List of species of genus *Frankia* proposed by Becking (1970). (Normand and Fernandez, 2006) (Prof. Phillippe Normand, University of Lyon, France kindly provides this figure).

Plant Order	Plant family	Host plant genus	*Frankia* species	Vesicles (size,shape)
Fagales	Betulaceae	*Alnus*	*alni* (type)	3-8µm, spherical
Rhamnales	Elaeagnaceae	*Elaeagnus*	*elaeagni*	2-4µm, spherical
"	Rhamnaceae	*Ceanothus*	*ceanothi*	1.5-3µm, spherical
"	Discariaceae	*Discaria*	*discariae*	4µm, sphaerical
Myricales	Myricaceae	*Myrica*	*brunchorstii*	7.5-12.5x1.6-2.4µm, club
Casuarinales	Casuarinaceae	*Casuarina*	*casuarinae*	3-4x0.6-1.5µm, club
Coriariales	Coriariaceae	*Coriaria*	*coriariae*	9-12x1.2µm, club
Rosales	Rosaceae	*Dryas*	*dryadis*	1.5-5x1.5-2µm, club
"	"	*Purshia*	*purshiae*	ND
"	"	*Cercocarpus*	*cercocarpi*	ND

(Fig 2.9) .

This classification was based on the finding that *Myrica* is a promiscuous host, which was infected by most of the *Frankia*, with exception with *Myrica gale*, this species can be nodulated by only *Alnus*-infective strains (Huguet *et al.*, 2005).

Lalonde *et al.* (1988) proposed another taxonomical scheme based on the system proposed by Lechevalier, but he used the names *alni* and *elaeagni* to refer to host plants. He further subdivided *F. alni* into two species namely *pommerii* and *vandickyi*, which was based on the spore positive (sp^+) and spore negative (sp^-) characters. But this idea was later discarded due to uncertainties on spore characters and

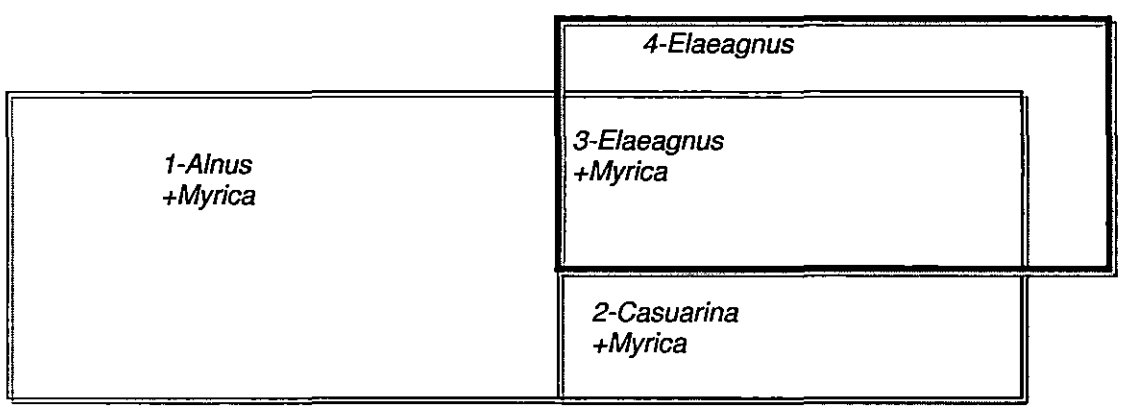


Fig 2.9 Schematic of strains grouping based on infection of host plants (Baker, 1987). (Prof. Phillippe Normand, University of Lyon, France kindly provides this picture). (Normand and Fernandez, 2006)

Table 2.4. Synopsis of strains tested for DNA homology (Fernandez *et al.*, 1989; Lumini *et al.*, 1996), and 16S sequence is available for phylogeny approaches. (Prof. Phillippe Normand, University of Lyon, France kindly provides this scheme). (Normand and Fernandez, 2006)

Species by Fernandez *et al.*, 1989	**Ref. strains**	**Other strains tested**	**16S available**	**Infectivity**
Genomic species 1	AcoN24d	CpI1, ArI3, $ACN1^{AG}$	ACN14a	*Alnus, Morella*
Genomic species 2	AV22C	AVN17o	AVN17s	"
Genomic species 3	$ARgP5^{Ag}$		$ARgP5^{Ag}$	"
Genomic species 4	Ea1-12	$HR27_{14}$, $Ea1_2$, $Ea2_6$, $Ea3_3$ $Ea1_2Ea33$	$Ea1_2$,	Elaeagnaceae, *Morella*
Genomic species 5	$TX31e^{HR}$	EAN1pec, HRX401a	EAN1pec	*Morella*
Genomic species 6	EUN1f			*Morella+Alnus*
Genomic species 7	HRN18a			*Morella+Alnus*
Genomic species 8	Ea50-1			*Morella*
Genomic species 10				*Morella+Alnus*
Genomic species 11				*Morella+Alnus*
Genomic species 12			SCN10a	*Morella*
Genomic species 9	CeD (ORS020606)	ORS020607, Cj1-82, AllI1	CeD, CcI3	*Casuarina*

absence of molecular evidence in favor of spore characters.

An *et al* published a series of papers on the use of DNA homology technique in classification of *Frankia* (An *et al.*, 1983; An *et al.*, 1985; An *et al.*, 1987;). This bacterium has a high G+C content of 68-72% (An *et al.*, 1983). Prof. An examined 19 isolates and proposed 1 species with 9 *Alnus*-infective isolates, 1 species with a single *Elaeagnus*-infective isolate and several unclustered isolates. The *Alnus*-infective isolates belong to the cluster 1 and the *Elaeagnus*-infective isolate belongs to the cluster 3 of this bacterium. Later Fernandez *et al.* (1989) worked in the same way with 43 isolates and proposed 9 *Frankia* species among them, in which 3

belongs to the group of *Alnus*-infective (i.e. belongs to cluster 1), 5 belongs to the group of *Elaeagnus*-infective (i.e. belongs to cluster 3) and the last one in the group of *Casuarina*-infective (also in cluster 1) strains (Tab 2.4). Dobritsa and her co-workers (Akimov *et al.*, 1991; Akimov and Dobritsa, 1992;) partially extended the study by Fernandez *et al.* and found 5 genospecies in the *Alnus* infective strains and four *Frankia* genospecies on *Elaeagnus* infective strains. However, in both the group tested few strains and therefore it is impossible to conclude if these numbers add to those previously described or not. The exception of this case is the *Frankia alni* strains CpI1 and ArI3. Lumini *et al* (1996) worked on DNA homology further with other *Elaeagnus*-infective strains, particularly with those strains that can infect both of *Elaeagnus* and *Alnus*, and they described three further species. These species have no pure culture existence and their differentiations are based only on DNA hybridization. They are called genomospecies or genospecies (Normand and Fernandez, 2006). Host infectivity or host species of origin are studied in this species. The *Casuarina* infective strains are closely homologous as evident by DNA homology values (above 70%; Fernandez *et al.*, 1989) and have a very conserved PCR-RFLP patterns in the *rrn* and *nif* operons (Rouvier *et al.*, 1992, 1996). When a *Frankia* strain is isolated from the *C. equisetifolia* out side Australia, the strains showed variations. Thus it can be concluded from the above discussion that there are about 3 to 7 or more genomospecies infective on *Alnus* and *Myrica* and from 8 to 12 or more genomospecies infective on Elaeagnaceae and *Gymnostoma*. So, a minimum of 12 species are available in isolates under the genus *Frankia*. So, it is possible to that there exists over hundred species of *Frankia* which are distributed in the four clusters.

Isolation of *Frankia* from Rosaceous members or from *Datisca* or from *Coriaria* is very difficult. According to Becking (1970) there were at least three *Frankia* species present in Rosaceous plants. Mirza *et al.* (1994) first showed that the *Frankia* isolated from *Datisca* could nodulate *Coriaria*, and both have the similar 16S rRNA sequence. He presumed that these bacterium infecting both *Datisca* and *Coriaria* may be the same bacterium. Bosco *et al.* (1994) on the basis of 16S

rRNA sequence concluded that the symbiont isolated from *Dryas* (a member of the family Rosaceae) are close to those of *Datisca* and *Coriaria* symbionts. These plants can also be nodulated by cluster 3 genomospecies under laboratory conditions. These cluster 3 strains are cosmopolitan and can cross infect with other actinorhizal plants while other strains are selective.

The prime obstacle of *Frankia* research lies in its difficulties in the isolation. With invention of polymerase chain reaction (PCR) technology (Mullis *et al.*, 1986) identification of *Frankia* become easier. First PCR amplification of *Frankia* was done with universal primer FGPS849 and FGPS1176 and the size of amplicon was 325bp (Nazaret *et al.*, 1991). The first 16s rDNA specific primers (FGPS989ac and FGPS989e) targeting the helix 31 of domain III of 16s rRNA gene was developed by Bosco *et al.* (1992). According to Misra and Verghese (2004) the ITS region of *rrn* genes shows good diversity among *Frankia* strains. Simonet *et al* (1991) and Mirza *et al* (1994) constructed primers for that region and successfully amplified the ITS region of *rrn* gene of *Frankia.* Normand *et al* (1994) studied the structure of *rrn* operon in *Frankia* and compared with corresponding gene of *Streptomyces* for taxonomic purpose. The length of 16s rDNA is 1513nt, 23s rDNA is 3099nt and 5s rDNA is 200nt in *Frankia.* The 16s rDNA region of *Frankia* is highly conserved (Misra and Verghese, 2004)

Hahn *et al* (1989) were the first to use 16S rRNA sequences of *Frankia* to investigate the phylogenetic relationship of *Frankia* with its neighbors. On the basis of their study it was revealed that *Frankia* was close to *Geodermatophilus*, a dry soil actinomycetes, in possessing similar multilocular sporangia and to *Blastococcus*, a sea microbe. But there is little description about the genus *Blastococcus* (Normand and Fernandez, 2006). Marechal *et al* (2000), based on their studies on, *recA* marker on this regard found an interesting finding that a thermal spring microbe, *Acidothermus cellulolyticus* is the closest phyletic neighbor of *Frankia* rather than *Geodermatophilus* . Recent studies also confirm that *Frankia* can be subdivided into four clusters (Fig .2.10 and 2.11)

Frankia is world wide distributed and faces various physiochemical soil properties like soil pH, aeration, moisture etc. Even this bacterium may

persist in soil in absence of the host plants (Huss-Danell and Frej, 1986; Smolander and Sundman, 1987). It has been found that actinorhizal plants gets nodulated far away from their original habitat. This indicates the presence of that particular *Frankia* in that area (Dawson *et al.*, 1989; Smolander, 1990; Jamann *et al.*, 1992, Huguet *et al.*, 2004, McCray Batzl *et al.*, 2004; Normand and Fernandez, 2006).

Jamann *et al.* (1992, 1993) working on *nifD-nifK* intergenic region of *Frankia* from *Elaeagnus* shows that the diversity which is measured by the Shannon-Weaver diversity index differ in accordance with soil pH. Soil pollutant also has a role in *Frankia* diversity, which was assessed by Ridgway *et al.* (2004) in *Frankia* strains associated with *Alnus incana.* It was found that different metal concentration play a major role in distribution of *Frankia* associated with *Gynmostoma* (Navarro *et al.*, 1999).

The recent whole genome sequencing data of the three strains of these bacterium shows that there are huge variation in the genome size (Fig 2.12). The *Frankia* strain EAN1pec has a genome size of 9,035,218,7976bp while the *Frankia* strain CcI3 has genome size of about 5,433,6284499bp. The *Frankia* strain ACN 14a has intermediate genome size of about 7,497,9346786bp (Normand *et al.*, 2007). Normand *et al.* (2007) found 2,810 genes common in the genome of CcI3, ACN14a and EAN1pec strain of *Frankia.* So it can be stated that the diversity of *Frankia* strain lies in their variable genome size. So the conclusion on the diversity of *Frankia* can be drawn from the word of Normand "The full genetic diversity of *Frankia* may not yet have been revealed due to limits brought about by the approaches used. Indeed, the time-consuming isolation step not only strongly limits sampling, it also induces biases, under representing non- or poorly nodulating strains and selecting those isolates that are easily culturable. A previous facilitating but bias-inducing step often seen is the use of an intermediate host inoculated in the laboratory by crushed nodules from the field. Indeed, it was reported that the genetic diversity observed in natural populations can be totally different from that observed following greenhouse inoculations with soil or field nodules (Huguet *et al.*, 2005). Direct detection has been used in several recent attempts to describe *Frankia* diversity in nodules or soil of

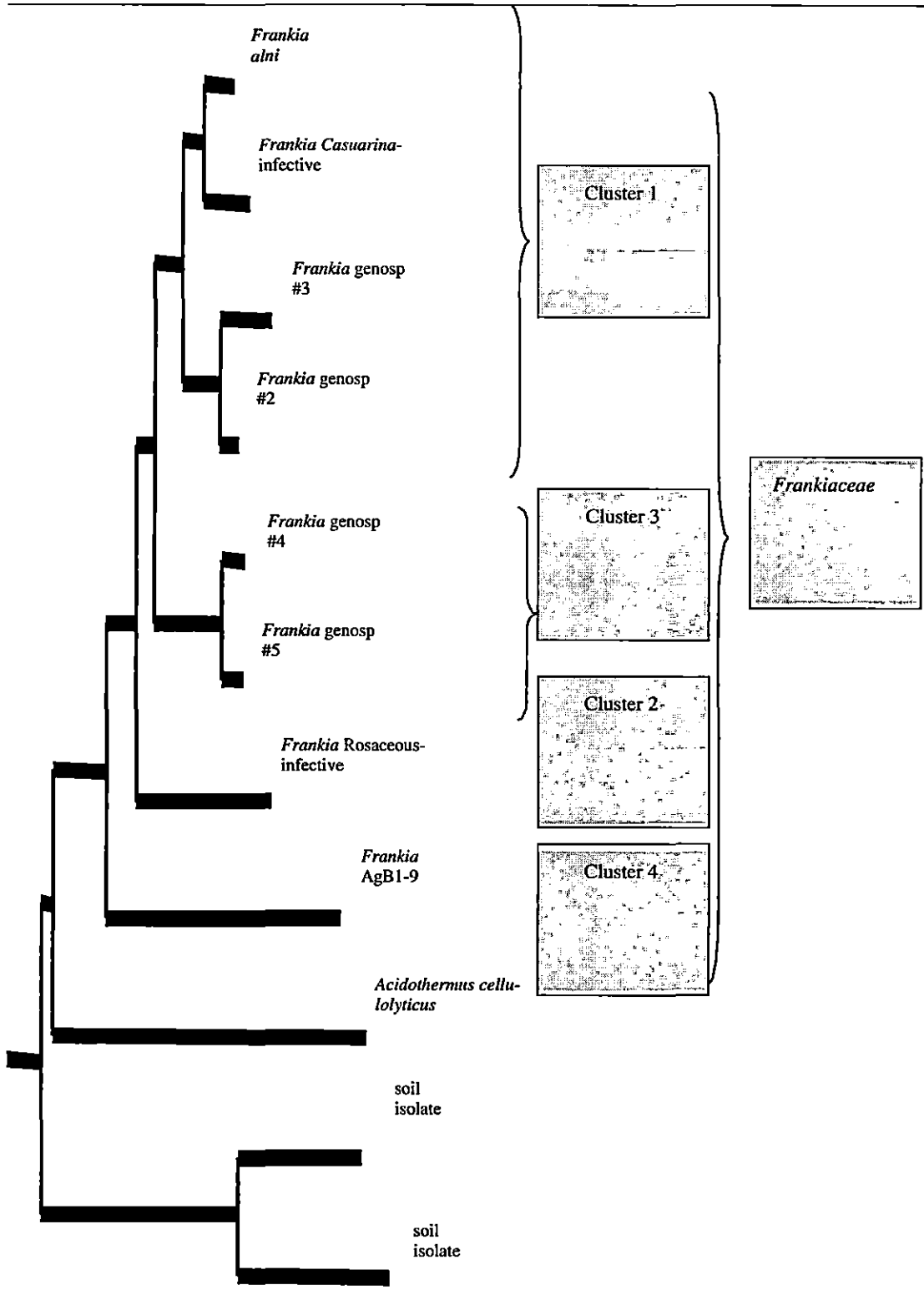


Fig 2.10: Phylogenetic tree (Saitou and Nei, 1987) of *Frankia* and closest neighbors comprising soil sequences. 1: *Alnus* and *Casuarina* infective strains; 2: *Rosaceaous-*, *Datiscaceae-* and *Coriariaceae*-unisolated symbionts; 3: *Elaeagnus*-infective strains; 4: a divergent group of ineffective or non-infective strains. (Normand and Fernandez, 2006)). ((Prof. Phillippe Normand, University of Lyon, France, kindly provides this dendogram)

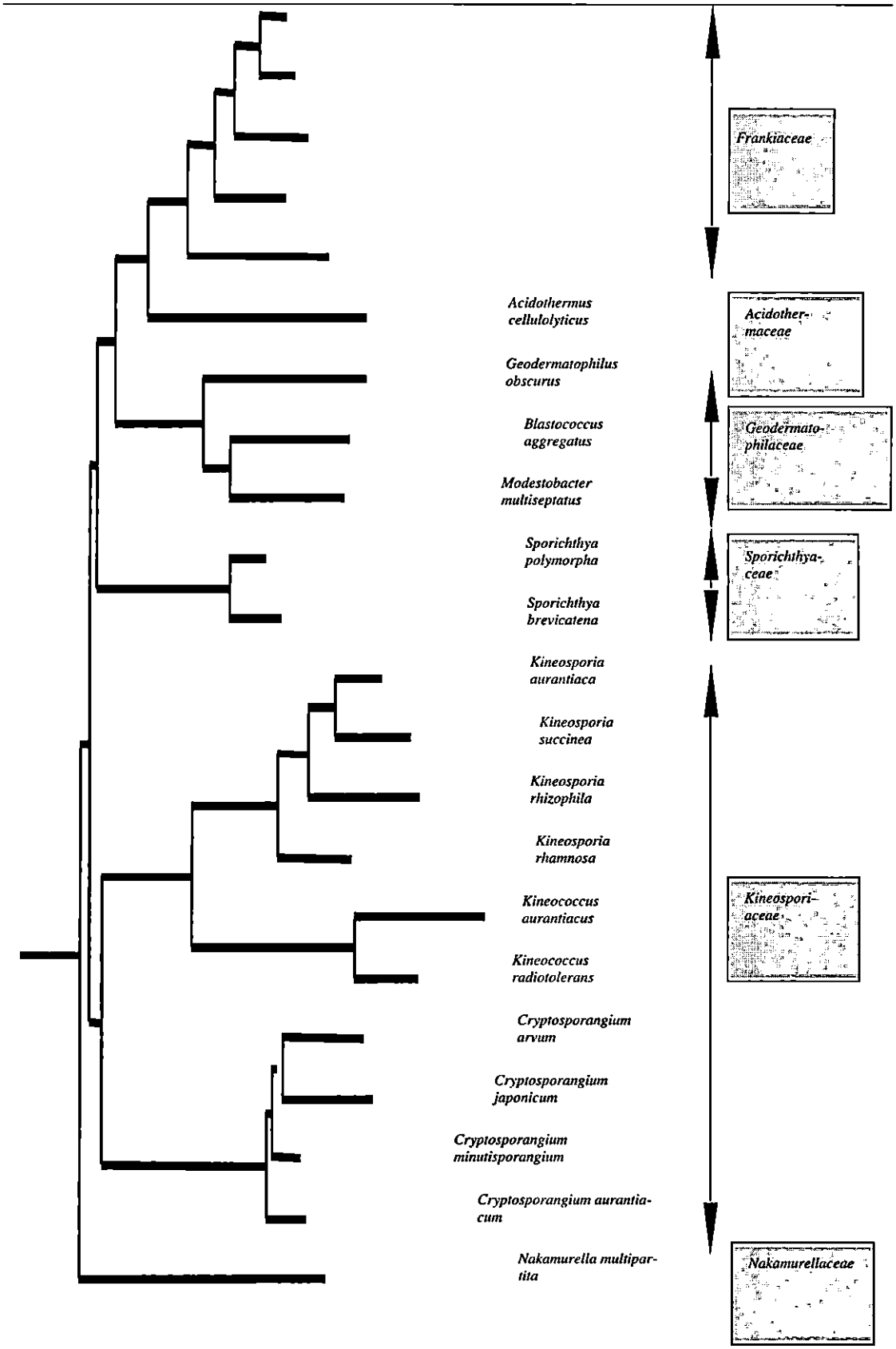


Fig 2.11: Phylogenetic tree of the Frankinae. by the Neighbor-Joining (Saitou and Nei, 1987) method including all validly described genera and species of suborder Frankinae (Prof. Phillippe Normand, University of Lyon, France kindly provides this dendogram). (Normand and Fernandez, 2006)

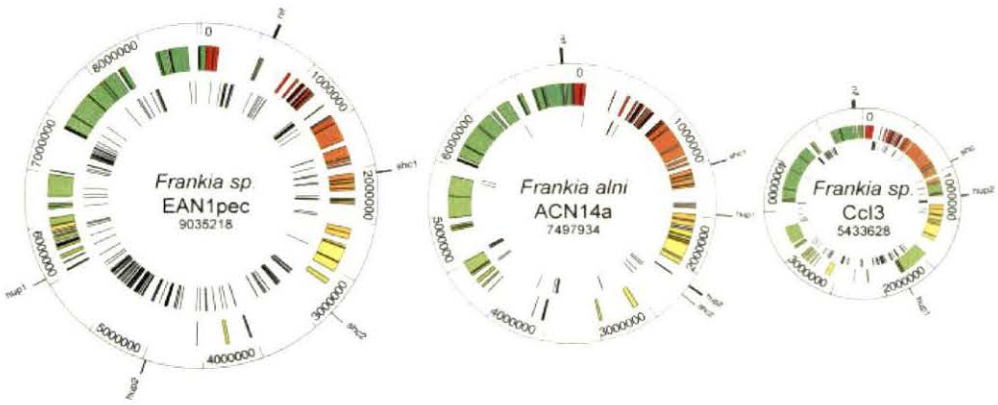


Fig 2.12 Genome maps of the three *Frankia* strains. Circles, from the outside in, show (1) gene regions related to symbiosis including *shc*1, *hup2*, *hup1*, and *nif*; (2) the coordinates in Mb beginning at 0 = oriC; (3) regions of synteny (syntons) calculated as a minimum of five contiguous genes present in all strains with an identity >30% over 80% of the length of the shortest gene (syntons are tagged with a spectrum-based [red-yellow-green] color code standardized on ACN to indicate regions where syntons have moved in the other strains); (4) IS elements and transposases. Circles were drawn using GenVision Software from DNAStar.(Adopted from 'Genome characteristics of facultatively symbiotic *Frankia* sp. strains reflect host range and host plant biogeography' Philippe Normand *et al*, 2007 Genome Res. 2007 January; 17(1): 7–15.

PCR-amplified DNA fragments, but these methods are often based on one or two conserved genes (generally *rrn* and *nif* genes) and cannot but fail to describe the full extent of biological diversity." (Normand and Fernandez, 2006). Thus according to the present taxonomic status of this bacterium belongs to the monogeneric family Frankiaceae (Normand *et al.,* 1996), which is a member of suborder Frankinae together with 5 other families namely Acidothermaceae, Geodermatophilaceae, Sporichthyaceae, Kineosporiaceae and Microsphaeraceae, and the suborder Frankineae is a member of the order Actinomycetales (Normand and Fernandez, 2006).

2.3.5 Bioinformatics of *Frankia*

Bioinformatics is essentially a marriage between biology, information technology and computer science. In the present era, bioinformatic analysis is virtually required in every aspect of bioscience and *Frankia* research is no exception. Perhaps the first *Frankia* partial sequence submitted to the public domain was a 243bp long sequence of rDNA (GenBank accession no S70679.1) done by Lyon group (Nick *et al.,* 1992). Since then on, several sequences have been submitted in various DNA depositories

like GenBank, EMBI database, DDBJ, etc. The first DNA sequence which appear from India was done by AK Misra group (GenBank: S70135.1).

Two groups came forward for *Frankia* whole genome sequencing project. These are LS Tisa and D. Benson from USA, who have done the sequencing of *Frankia* strains CcI3 and EAN1pec in collaboration with DOE, JGI, USA and P Normand, who did the sequencing of *Frankia* strain ACN14a with Genoscope, France. With the advent of *Frankia* genome project various other workers started working on bioinformatics of *Frankia*. Prominent among them are Sen and his group in University of North Bengal, Ventura *et al.* (2007) and McEwan and Gatherer (1999). McEwan and Gatherer (1999) first used codon indices to predict the expression of *nif* operon of *Frankia*. Sen and his group published a series of paper on bioinformatics of *Frankia* (Sur *et al.*, 2006, 2007; Bose *et al.*, 2007; and Sen *et al.*, 2008, 2010). They used certain parameters like GC content, GC3 content (G or C nucleotide in the third codon position), effective number of codon (Nc), relative synonymous codon usage (RSCU), Fop (frequency of optimal codon) and CAI (codon adaptation index) to investigate codon usage pattern of whole genome of *Frankia* with special reference to *nif* genes. Sur *et al.* (2006, 2007) studied the codon usage profiling and analyze the intergenic association of *Frankia* EuIK1 *nif* genes and compared the result with *Bradyrhizobium*. On the basis of codon usage data they concluded that *Frankia* strain show high level of codon preference. In another study (Sur *et al.*, 2007), this group found that the expression level of *nif* genes of *Frankia* are moderately high and show mutational bias. The life style pattern of various *Frankia* strains was studied using codon usage pattern analysis (Sen *et al.*, 2008). It was found that *in Frankia*, the highly expressed genes are more biased in comparison to the other protein coding genes. The analysis of COG profiles of the predicted highly expressed genes further highlighted the differences amongst the *Frankia* strains. The results obtained in their study further supported the hypothesis that *Frankia* CcI3 is slowly becoming a symbiotic specialist. Besides such sequence analysis studies, structurally analysis of proteins of *Frankia* have also been carried out. The three-dimensional structure of NifH protein of *Frankia*

was resolved using homology modeling technique (Sen *et al.*, 2010). The metal binding sites and important functional regions of this protein have also been determined. The structure provided valuable insight into the 3D framework and structure-function relation of this protein. Beside *nif* genes other genes of *Frankia* also got serious attention from the workers. Kosawang (2009) studied the regulation of hydrogenase using comparative genomics of *Frankia* strain CcI3, ACN14a and R43. *Frankia* strain R43 show an interesting feature in its hydrogenase protein. It works in bidirectional mode and evolved hydrogen which is absent in CcI3, ACN14a. In this decades the knowledge and technologies of structural bioinformatics, protrome and secretome research was generated (Sur *et al.*, 2010) which gave a boost in understanding the biological nitrogen fixation by *Frankia*. The proteome of *Frankia* was first studied by Alloisio *et al.* (2007) in both under nitrogen fixing and nitrogen depleted condition. This study reveals that total of 126 proteins is associated with nitrogen assimilation and oxidative defense system. Mastronunzio *et al.* (2008) studied the secretome of *Frankia* and unable to locate any polysaccharide degrading enzyme within it. They attributed property of secretome to a group of esterase, lipases and hydrolases which help in the process of nodulation. Niemann and Tisa (2008) found two truncated hemoglobin genes (*hboN* and *hboO*) in *Frankia* strain CcI3 genome. These genes help in the oxygen protection in *Frankia* (Misra and Sen, 2011). Mastronunzio and Benson (2010) used mass spectroscopy and two dimensional liquid chromatography in proteome research in *Frankia* and identified 1300 proteins of *Frankia* isolated from *Alnus incana*, 1,100 proteins from *Elaeagnus angustifolia* and 100 proteins from *Ceanothus americanus*. They found that the iron proteins are the most abundant proteins in *Frankia*

Recently Sen *et al.* (2011) explored the significance of rare TTA codon containing genes in *Frankia*. Amongst the high GC content genomes of Actinomycetes, *Frankia* genomes had the highest percentages of TTA containing genes. They have found that *Frankia* genomes retain large number of such genes in several important functional groups such as metabolism and cellular process. Many of these genes had ortholog in other *Frankia*

genomes. Their study on evolutionary significance and codon-anticodon interaction further point to the fact that so many of the *Frankia* genes hold on to this rare codon without compromising their translational efficiency.

The study of morphology and physiology of *Frankia* was started from the time of first isolation. In the last decade of the last century genetics and molecular biology based work of *Frankia* started and gathered a good chunk of information about this organism, which reached its peak with whole genome sequencing of the organism. In the recent past post sequencing work using bioinformatics tools started and *Frankia* research grew up. However, many opportunities of study have been left in this field, which invites serious attention of workers.

Chapter 3
MATERIALS & METHODS

3.1 Isolation and Identification of *Frankia*

3.1.1 Survey of Actinorhizal Plants

The present study included two actinorhizal plants growing in West Bengal. These are *Alnus nepalensis* D.Don and *Casuarina equisetifolia* L. *A. nepalensis* is common in the hills of Darjeeling district (Eastern Himalayas) at an altitude between 1670 and 2040 m (up to 3000 m) with a temperate climate (Sharma and Ambasht,1986). It prefers moist, cool climate with mean annual temperature of 13-26°C. On the other hand, mature *C. equisetifolia* is common plant in sea shore. It is cultivated all through the sea shore of South India, Odisha and West Bengal. In West Bengal this tree is very common in Midnapur and South 24-Pargana district. However, scattered plantation can be found in all over the state, particularly in Kolkata, Howrah, Hooghly and North 24 pargana district.

3.1.2 Collection of Germplasm

Germplasm were collected from different places of West Bengal, which includes the coastal as well as hilly regions of the state. There are two actinorhizal plants present in this region- *A. nepalensis* and *C. equisetifolia*. *A. nepalensis* (up to 3000m) is growing in the hilly regions of the Darjeeling district and *C. equisetifolia* is mainly growing in the coastal regions. Although *C. equisetifolia* can be seen in the other parts of Bengal in planted condition and they are growing luxuriantly.

Nodules (germplasm for *Frankia*) of the *A. nepalensis* samples were collected in the way of Siliguri – Darjeeling – Siliguri route via Mirik-Pasupati- Ghoom- Sonada. This plant is found in abundance as road side tree,

while *C. equisetifolia* germplasm were collected from several places like North Bengal University, Siliguri Teesta barrage campus, Kolkata and surrounding areas, Diamond harbor, Namkhana, Digha and Bok-khali. The fresh, light whitish brown colored, young nodules were collected from mainly young healthy trees. The light colored nodules indicate the active growth and youngness (Myrold, 1994). Essential field data of the tree and the locality were collected in data collection sheet (Fig 3.1) which includes habit, habitat, vegetation in surrounding areas, soil type and nodules. Photographs of the plants and surrounding vegetations were taken (Fig 3.2 & 3.3). The vegetation were studied and recorded in a tabular form. The percentage of nodulation was calculated by the following formula of Raman and Elumalai, 1991:

$$\%\text{ of nodulation} = \frac{\text{No. of plants with nodules}}{\text{No. of plants observed}} X100$$

The collected nodules were stored in plastic bags containing moist tissue papers for the maintenance of active water potential and kept in ice box to minimize the tissue degradation. The soil samples were also collected in the same manner and both the samples were kept in -20°C for further use.

3.1.3 Isolation of the Bacterium:

Procedure I: Direct Isolation

In the first procedure nodules were collected from different places of West Bengal. For surface sterilization, the collected nodules were subjected to the treatment of 1%$HgCl_2$ and 30%H_2O_2 for different time span. The $HgCl_2$ treatment lasted 1 minute to 5 minute , and with 30% H_2O_2, it was for 10 minute to 30 minutes. The effective $HgCl_2$ treatment was 1 minute and it was 20 minute for 30%H_2O_2. After the treatment the nodule lobes were washed with sterile distilled water for several times. After final wash nodule lobes were peeled off with two sterile needles. Each lobe was then washed twice with sterile distilled water and was crushed in a 100 mL bottle with 25 mL of Define propionate minimal media (DPM-nitrogen free) (Baker and O'keefe, 1984)) without nitrogen.

Procedure II: Isolation of *Frankia* In Alginate Beads

In this procedure nodules were sterilized as per protocol standardized by Sarma *et al* (1998). About 0.5 grams of nodule lobes were taken in to the tissue homogenizer with 10mL of 3% PVP in phosphate buffer saline (PBS). The nodule lobes were crushed

Data Collection Sheet.

Sample no- MR 5

A. General Information-

1. Collection Site: Munik
2. Date: 23.3.2005
3. Time: 12.45 pm.
4. Scientific name of the host: Alnus nepalensis
5. Local name: Oothis
6. Nodule collected: Yes/No.
7. Root collected: Yes/No.
8. Seeds collected: Yes/No.
9. Soil collected: Yes/No.
10. Twig collected: Yes/No.

B. Habits:

1. Tree/Shrub/Herb
2. Flowering time:.....
3. Seeding time:.....
4. Planting time:.....

C. Habitat and area of the vegetation:

1. Rain fall season: From March to Oct-
2. Altitude (mt): 1500
3. Temperature: (°C) (a) Soil: 13°c (b) Air: 15 °c
4. Topography: Swamp/ Plain/ Hilly/ Others
5. Vegetation type: Natural forest/ Road side/ Social forest/ others
6. Management: Cutting/ Burning/ Natural/ Habitat preserved.

D. Specific collection site:

1. Site cover: Bear up to 20%/ 21-40%/ 41-60%/ 61-80%/ 81-100%.
2. Soil pH: 5.9
3. Color of the nodule: Red/ Yellow/ Reddish brown/Gray.
4. Drainage: Flooded/ poorly drained/ well drained.

E. Nodules:

1. Location: Crown area/ Tap root/ Lateral root.
2. Growth form: Clumped/ Scattered.

Collected by
Debadin Bose
Research Scholar, Molecular Genetics Lab.
Department of Botany, NBU.

Fig 3.1. A sample Data Collection Sheet.

Fig 3.2 A. *Casuarina equisetifolia* plant.
Fig 3.2 B & C. Male flower of *C. equisetifolia.*
Fig 3.2 D. Female flower of *C. equisetifolia.*
Fig 3.2 E & G Collection sites of *C. equisetifolia.*
Fig 3.2 F Nodules at the base of the *C. equisetifolia.*

Fig 3.3 *Alnus nepalensis* branches bearing fruits

properly and the debris were allowed to settle down for 10 min. The upper aqueous phase was then taken in a sterile conical flask and kept over night in a refrigerator to get rid of the phenolics present in the nodules (Leechvalier and Leechvalier, 1990). Next day, 1g of Na-alginate was dissolved in 40mL of liquid medium by keeping it in boiling water bath. Along with this, 250mL of 1% $CaCl_2$ solution was also prepared. Both the solutions were sterilized by autoclaving for 20 min at 1.1kg/cm^2. Then the aqueous phase of the crushed nodules was mixed with Na-alginate when the temperature of the solution cames down below 30°C. They were then dropped aseptically through a sterile syringe into the sterile $CaCl_2$ solution and kept for half an hour in the refrigerator for hardening. The beads were then harvested and washed with distilled water and finally with fresh medium. The beads were then distributed into aliquots with fresh medium.

Procedure III

In this procedure, fresh, young and light brown colored root nodules from mature actinorhizal plants were collected. The woody nodule lobes were surface sterilized with a series of surface sterilizing agents like 4% sodium hypochlorite solution for 7 min, 70% ethanol for 2 min, 0.1%

mercuric chloride solution for 7 min and finally in 30% hydrogen peroxide for 30 min in dark. Each step was followed by repeated washing with sterile distilled water. The nodule lobes were then incubated with Qmod (Lalonde and Calvert, 1979) medium supplemented with 3% activated charcoal for 48h at 30°C. After incubation, the nodule lobes were washed with sterile distilled water to remove charcoal. Sterilization process was repeated once again. Epidermal layers were removed and nodule lobes were chopped into small pieces. Each nodule piece was incubated with 10mL of Qmod (Lalonde and Calvert, 1979) medium supplemented with Vit B_1 and B_{12} in 30 mL screw cap test tube at 28°C for one month or more in dark without shaking (Bose and Sen, 2006).

3.2 Media for Isolation and Maintenance

Even a very complex isolation medium for *Frankia* is available, a variety of medium were tested. These were Defined propionate minimal media (DPM) (Baker and O Keefe, 1984), F-medium (Simonet *et al.*, 1985), OS-1 medium (Dobritsa and Stupar, 1989) and Qmod medium (Lalonde and Calvert, 1979), but the best medium for isolation of *Frankia* beside *Frankia* isolation medium is the complex Qmod (Lalonde and Calvert, 1979) medium. Define propionate minimal medium is also a very good medium for maintenance of the endophytes in long term.

3.3. Maintenance of Pure Culture

The pure cultures were maintained in both minimal and complex medium and named accordingly, *i.e.* first two letters from the genus and the species of the host plant with the first one capitalized and the subsequent two letters from the collection site. For example the strains isolated from Siliguri and adjoining area from the nodules of *C. equisetifolia* were named as CeSi1, CeSi5 etc. Similarly the strains isolated Mirik and adjoining areas from the nodules of *A. nepalensis* were named as AnMr1, AnMr2, etc. The pure cultures were maintained in DPM (Baker and O'keefe, 1984) and Qmod (Lalonde and Calvert, 1979) medium in 100mL screw cap bottles in 10mL of requisite medium and sub culturing were done in every 2-6 months. The *Frankia* cultures grow best in liquid culture between 25^0 to 35^0C. New stains often slow grower than once that have been in culture for some times and strains can be adopted to grow more rapidly by frequent transfers. The hyphae were fragmented

by passing throw a sterile needle. The fragmentation helps in the faster growth of this bacterium. The culture characteristics of the bacterium were also observed.

3.4 Plant Infectivity Test

3.4.1 Germination Of Seeds

The seeds of the two hosts were collected from healthy host plants from the field conditions. The seeds of *A. nepalensis* were collected in the month of March from hills of Darjeeling districts and the seeds of *C. equisetifolia* were collected in the month of May from Namkhana, Kanning and Diamond harbor. Both the seeds need two different conditions for germination.

A. nepalensis seeds were pretreated with aerated water for over night and *C. equisetifolia* seeds needed no such pretreatment and germinate directly (Myrold, 1994). The seeds were taken separately in two different sterile conical flasks and surface sterilized with 30% H_2O_2 for 10 minutes and washed with sterile distilled water several times.

Mature seeds of *Alnus* were used in the study of the effect of different hormones and media in germination condition. Two treatments of seeds- overnight soaked with aerated water and non-soaked were used for the experiment. Three different media woody plant medium (WPM, Hi-Media, Cat#PT105) (pH-5.6), Murashige and Skoog medium (MS) (Hi-Media, Cat#PT0018) (pH-5.6) and Hoagland solution (pH-7) (Hoagland and Arnon, 1950) in half and full strength were used in the study to see the effect of media composition. 1 mL of media was poured in each culture tube carrying a strip of filter paper and was autoclaved at 121°C for 20 min at 1.08 Kg/cm^2 pressure. A few surface sterilized *Alnus* seeds were placed on the filter paper in the culture tube and were incubated at 25±1°C under white fluorescent light. For the study of effect of hormones in seed germination, the media were separately supplemented with 1-5mg/L NAA and IBA. Sterilized seeds were also placed on sterile vermicompost for germination and growth. Double layer culture media were prepared by pouring Hoagland medium (0.4 % agar) on MS medium (1.2 % agar), WPM (1.2 % agar) and Hoagland medium (1.2 % agar) supplemented with hormones as described earlier.

The *C. equisetifolia* seeds were kept in a sterile plastic box in free floating condition in BOD incubator at 26 ±

3^0C for germination.

Fifteen day to one month old seedlings were then placed in sterile blotting paper supported by stainless steel supporter in magenta box (Planton, Tarsons make) containing sterile Hoagland Solution without nitrogen of various concentrations (1/2, 1/4, 1/8, 1/16).

For the plant infectivity tests seedlings were inoculated with fresh crushed nodule suspension and treated it as +ve control. Seedlings were inoculated with 100µL of 30 days *Frankia* culture under examination. A set of uninoculated seedlings were used as – ve control. The seedlings were kept in plant growth chamber at 26^0C with approximately 90% humidity and 1100 lux illumination.

3.5 Field Emission Scanning Electron Microscopy

For field emission scanning electron microscopy, one month old *Frankia* cultures were used. The bacterial cells were taken in a micro centrifuge tube and centrifuge at 1000 rpm for 5 minute. The media was then discarded and cells were washed with distilled water. The bacterial mycelium were treated with 4% glutaraldehyde solution in phosphate buffer (pH 7.2) for 4h at 30^0C. The glutaraldehyde solution was decanted and the samples were washed with the same buffer for three times. The samples were treated with 1% Osmium tetra oxide solution in phosphate buffer (pH 7.2) for 1 hour and washed with the phosphate buffer (pH 7.2) for three times. The *Frankia* samples were passed through with graduated ethanol from 50% to absolute for 10 minute each for dehydration. The 95% ethanol wash was performed twice. After dehydration the samples were kept in absolute alcohol for microscopy (Sen, 1996; Bajwa, 2004)

The samples were critical point dried for 2h. Each sample was mounted on copper tape and was platinum coated by JFC 1600 platinum caster, (JEOL, Japan) and viewed on JEOL JSM-6700F field emission scanning electron microscope at an accelerating voltage 2kV.

3.6 Physiology of *Frankia*

Eleven *Frankia* strains were selected and used in the study of physiology of the bacterium. The reference strain used in this study was $ACN1^{AG}$ which was kindly provided by Prof. Louis S Tisa, Department of Microbiology, University of New Hampshire, USA. *Frankia* strains were characterized on the basis of organic acid

decarboxilation, protease and β-glucosidase (Horriere, 1984; Weber *et al.*, 1988) activities as well as utilization of twelve different carbon sources.

3.6.1 Decarboxylation of Organic Acids

Decarboxylation of organic acids were determined as described by Leechvalier *et al.* (1983). Organic acid decarboxylations were determined in a basal medium containing in gL^{-1}: peptone, 5; NaCl, 1; $MgSO_4.7H_2O$, 0.2; KH_2PO_4, 0.2; $CaCl_2.2H_2O$, 0.01; Fe Na EDTA,0.01 and phenol red indicator (0.04% sol) 20mL L^{-1}. The pH was maintained at 6.8. The sodium salts of organic acids were added at 0.2% prior to the autoclaving. The organic acid salts were Na-citrate, Na-propionate, Na-acetate and Na-pyruvate. Decarboxylation leads to a rise in pH and assessed as change in color of the indicator from yellow - orange to red. 5mL of the sterile decarboxylation medium was inoculated with 1mL of thick hyphal suspension of *Frankia* and incubated at 28°C in dark for 42 days. The tubes were checked every alternate day.

3.6.2 Gelatin Hydrolysis Test

The protease activity of the *Frankia* strains were tested with gelatin as enzyme substrate. DPM (N_2 free) (Baker and O'keefe, 1984)) was supplemented with 12% of gelatin and 5mL of such sterile medium was inoculated with 1mL of thick hyphal suspension of *Frankia* and incubated at 28°C in dark for 42 days. The tubes were checked every alternate day.

3.6.3 Esculin Hydrolysis Test

A five week old *Frankia* suspension was inoculated in 5mL of DPM (Baker and O'Keefe, 1984) medium agar tube supplemented with 0.1% ferric citrate and 0.1% esculin. The esculin was hydrolyzed to a β-glucosidase activity and gave rise to coumarine. This coumarine react with ferric citrate to form a brownish black complex. The tubes were incubated at 28°C in dark for 42 days and checked every alternate day.

3.6.4 Utilization of Different Carbon Source

For utilization of single carbon source as source of energy the basal salt solutions were as same as the DPM (Baker and O'Keefe, 1984) medium (without nitrogen and propionate). The carbon source was added to this basal medium separately at 20 gL^{-1}. The carbon sources were glucose, sucrose, fructose, lactose, mannitol, Na-acetate, Na-propionate, Na-succinate, Na-pyruvate, Na-citrate and tween 80.

Tween 80 was added at 1mL L^{-1}. 5mL of basal medium was supplemented with the carbon source as stated above. A five week old *Frankia* culture was taken and the colonies were collected by centrifugation (at 12000rpm, for 2 min) and the colonies were crushed in 1.5 mL of sterile distilled water into a hyphal suspension. Each tube of basal medium supplemented with the carbon source was inoculated with 50µL of *Frankia* hyphal suspension. The tubes were incubated at 28°C in dark for 60 days to see the visible growth. The media pH was maintained at 7. The results thus obtained were analyzed with the free software POPGENE to calculate the diversity among the *Frankia* strains by Nei genetic diversity (Nei, 1987), Shanon index (Shannon, 1948; Weaver and Shannon, 1949), single locus component and two locus component (Brown and Feldman, 1981) and Wahlund effect (Brown and Feldman, 1981). All the above experiments were done in triplicate and same results were found. (Bose *et al.*, 2011)

3.7 Heavy Metal Salt Resistance Pattern

Frankia cultures were subjected to heavy metal stress to determine its sensitivity by growth inhibition assay. The heavy metal salts included nickel chloride ($NiCl_2$), cadmium chloride ($CdCl_2$), lead nitrate ($Pb(NO_3)_2$), cobalt chloride ($CoCl_2$) and copper sulphate ($CuSO_4$). The metal salts were used at the concentration of 0.005, 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2, 3, 4 and 5 mM. Two week old culture was used as inoculum. The hyphae were collected by centrifugation at 10,000 rpm for 2 min and fragmented by passing through an aseptic syringe. 2% DPM (Baker and O'keefe, 1984) agar plates containing various heavy metal salts at different concentrations were prepared separately. Half (1/2) mL of hyphal suspension was mixed with 5 mL of 0.8% sterile water agar at 45°C. The hyphal suspension mixed with water agar poured uniformly on 2% DPM (Baker and O'keefe, 1984) agar plates containing various heavy metal salts at different concentrations separately. Before pouring the content of the tube were mixed by agitation using a vortex mixer, but vortexing should be done carefully to avoid foaming in the agar. Plates were incubated at 28°C in dark for 14 days.

Colonies of *Frankia* appearing on the plates were taken as resistant and counted. The colony count was plotted against the increasing concentration of various heavy metals.

The main advantages of the double layer technique over the liquid culture media are as follows:-

1. Contaminants do not necessarily over grow the *Frankia* colonies.
2. Microaerophilic *Frankia* colonies are not directly exposed to the atmosphere.
3. *Frankia* colonies can be detected under microscope or even under naked eye.
4. Colony morphology of *Frankia* can be done. (Bose and Sen, 2005, 2006)

3.8 Analysis of Heavy Metal Resistance Genes of *Frankia*

The genome sequences of the three strains of *Frankia,* namely ACN14a, CcI3 and EAN1pec, were available (Normand *et al.,* 2007). They showed a degree of homogenecity in the 16s rRNA sequence (98%-99% identity), however the size of these genome varies significantly. These variations mainly arise due to the result of gene duplication and gene deletion (Normand *et al.,* 2007). These gene duplication and gene deletion are associated with host diversity, biogeographic location of the strains and its their life cycle pattern (Sur *et al.*, 2008). From these whole genome sequence data of *Frankia* strains it was evident that the strain EAN1pec had an expanding genome which helps the bacterium to adopt in new niches and with new substrates with its host. On the other hand the strain CcI3 with its reducing genome became more specialized to a particular environment in terms of host and substrates.

Codon usage and codon preferences are the interesting tools for studying the variations among these kinds of organisms (Grantham *et al.,* 1981; Karlin *et al.*, 2001, Sur *et al.*, 2008). The G + C composition variations are the results of either translational selection or mutational bias or both and it fix the path of codon variation (Knight *et al.*, 2001). The highly expressed genes are greatly affected by translation selection and show higher degree of biasness in the codon usage pattern (Ikemura, 1981; Sharp and Li, 1987; Dos Reis *et al.,* 2003; Banerjee *et al.*, 2004). The codon usage is nonrandom and non specific and in microorganisms, it is produced by directional mutational pressure and natural selection acting at the level of translation (Gouy and Gautier, 1982). The strength and direction of these forces fluctuate within and across the organisms; depending on their life style and genomic G+C content. The effective number of codon (Nc)

(Wright, 1990) and codon adaptation index are alternate measures of such biasness in organisms. The codon adaptation index (CAI) is a relative measurement of a gene with a particular set of reference gene, that also denotes the biasness at mRNA level (Ikemura, 1981; Sharp and Li, 1987; Dos Reis *et al.*, 2003; Wu *et al.*, 2005).

The heavy metal resistance system is an important survival strategy for the bacteria. This system arose soon after the life began (Silver and Phung, 1996) and attained a good diversity. The aim of this study was to explore the diversity of the heavy metal resistant gene, their codon use pattern and predict the expression level of that gene in comparison with protein coding and ribosomal protein genes.

The complete genome sequence of three *Frankia* strain were obtained from IMG website (http://www.img.jgi.doe.gov) (Markowitz *et al.*, 2006). The GenBank accession number of the strain EAN1pec is NC 009921, ACN14a is NC 008278, and CcI3 is NC 007777. At first a multiple sequence alignment was performed using CLUSTAL W taking the nucleotide sequences of the studied heavy metal résistance genes and a dendrogram was constructed to find out the diversity amongst them. All the protein coding genes, ribosomal protein genes, and heavy metal resistances were analyzed by the software CodonW (Ver. 1.4.2) (http://www.molbiol.ox.ac.uk/win95.codonW) and CAI calculator 2 (http://www.evolvingcode.net/codon/CalculatorCAIs.php). The effective number of codons (Nc) was calculated as per Wright (1990). This index is a simple measure of overall codon bias. Its value represents the number of equal codons that would generate the same codon usage bias that was observed. Under random codon usage the expected value of Nc was calculated by the following formula of Wright, (1990):

$$Nc=2+S+\{29/[/[S^2+(+(1-S)^2)^2]\} \quad \ldots\ldots\ldots (1)$$

Where, S represents GC3 values.

A commonly used measure of codon bias in prokaryotes and eukaryotes is the codon adaptation index (CAI). It is a measurement of relative adaptedness of a gene's codon usage towards the codon usage of highly expressed genes. The relative adapted-ness (ω) of each codon is the ratio of the usage of each codon, to that of the most abundant codon within the same synonymous family.

CAI (Sharp and Li, 1987) is calculated as follows:

$$CAI = exp\left(\frac{1}{L}\sum_{k=1}^{L} \ln \omega k\right) \quad ...(2)$$

Where, ω_k is the relative adapted-ness of the k^{th} codon and L designate the number of synonymous codons in the gene.

The software CodonW was developed by Peden (1999) which is used to calculate the GC3 and effective number of codon. The GC3 denotes the number of guanine and cytosine in the third position of the codon and the effective number of codon produce a overall picture of codon biasness. In highest conserved organism the value of effective number of codon is 20. That indicates the use of a single codon for a particular amino acid, when other alternative options are available. In highest biased case the value is 61. It signifies that the particular organism is utilizing all the codon equality. The codon adaptation index or CAI is a measure of codon uses within a gene relative to a reference set of genes (Sharp and Li, 1987). Its value ranges from 0 – 1. The high value indicates that the expression of the gene of interest is similar to the reference set of gene, which is a generally ribosomal protein gene.

3.9 Isolation of Genomic DNA of *Frankia*

3.9.1 Isolation of Genomic DNA From Pure Culture

Following protocol (Bajwa, 2004) was followed for isolation of genomic DNA from pure culture of *Frankia* :

- One mL of 1 month old *Frankia* culture was taken in a micro centrifuge tube.
- The tube was centrifuged at 10000rpm for 10 minutes and the supernatant was discarded.
- The pellet was resuspended in 1mL of TE buffer (pH 8) (see appendix for composition) and transferred to a 1.5mL microcentrifuge tube (Tarsons, India).
- It was centrifuged at 10000rpm for 15 minutes and the pellet was dissolved in 1mL of TE buffer (pH 8.0)
- The solution was forced through 5mL sterile syringe needle of 0.56 X 25mm to break the colonies.
- To this solution 10mg/mL of molecular biology grade lysozyme (Sigma, USA. Cat#L-6876) and a pinch of acromopeptidase (Sigma, USA. Cat#A3422) were added and was incubated at 20^0C for an hour.
- 250µL of 20% SDS was added to this solution and it was incubated at

60^{0}C for 30 minutes and at room temperature for 15 minutes.

- The solution was then divided into two equal parts of 625µL each.
- To each tube equal volume of molecular biology grade equilibrated phenol (SRL Cat# 1624262) was added and mixed gently.
- The tubes were then centrifuged at 10000rpm for 10 minutes and the upper aqueous phase so obtained was taken to a fresh tube.
- An equal volume of chloroform (600µL) (E Merck Ind Ltd. Cat#822265) was added gently and centrifuged at 10000rpm for 5 minutes.
- The upper aqueous phase was taken in a fresh tube and to it 360 µL of isopropyl alcohol (E Merck Ind. Ltd. Cat # 17813) was added and the tube was kept in room temperature overnight.
- The tubes were then centrifuged at 4^{0}C for 20 minute at 12000rpm The supernatant was discarded.
- The pellet was washed with 70% ethanol.
- Finally the pellet was dried in vacuum desiccators and the DNA was resuspended in 10µL of pyrogen free water.

3.9.2 Isolation of Genomic DNA of *Frankia* From Host Nodules

Five *A. nepalensis* and five *C. equisetifolia* trees were chosen from different collection sites. The *A. nepalensis* trees were selected from different locations of Darjeeling hills. Plants were selected from Mirik (A1), Fatak (A2), Sukhiapukhuri (A3), Ghoom (A4) and Sonada (A5). *C. equisetifolia* were selected from Jalpaiguri (C1), North Bengal University Campus(C2), Diamondharbor(C3), Bokkhali (C4) and Digha (C5) region of south 24-Pargana district.

Procedure

- About 0.5 grams of tap water washed nodule tissue were taken in a 100mL beaker.
- The nodule tissues were incubated with 10mL of TE buffer supplemented with 2% activated charcoal for 24h.
- After incubation the tissues were taken out from the TE buffer and washed with distilled water and dried properly with a paper towel.
- The nodule tissues were grinded into a fine powder using liquid nitrogen and a mortar and pestle. Liquid nitrogen facilitates the grinding at extremely low

temperature (- 196^{0}C).

- Transfer the powder in to 15 mL of prewarmed (at 65^{0}C) DNA extraction buffer (see appendix II for composition).
- Incubate it for 1hr in 65^{0}C.
- Add equal volume of chloroform: isoamyl alcohol (24:1) to it and mixed it properly. The mixture was centrifuged for 10 minutes at 6000 rpm.
- Take the upper phase to a fresh tube.
- To this tube, 0.6 volume of ice cold isopropanol was added and stored at -20^{0}C over night.
- In the next day DNA was precipitated at 10,000 rpm for 10 minutes.
- The pellet was dried and dissolved in 500µL of TE buffer and transfer in to 1.5 mL centrifuge tube.
- Equal volume of phenol: chloroform: isoamyal alcohol (25:24:1) was added to the tube and mixed properly. The tubes were centrifuged at 10,000rpm for 10 minutes and upper phase was taken in a fresh tube.
- Equal volume of chloroform: isoamyl alcohol (24:1) was added to the tube and mixed properly. The tubes were centrifuged at 10,000 rpm for 10 minutes and upper phase was taken in a fresh tube.
- In this tube, double volume of ice cold ethanol and 100µL of Ammonium acetate (10M) (Merck, Cat no#61750105001730) was added and the mixture was incubated for 2hr in -20°C and centrifuge for 12,000 rpm for 30 minutes at 4°C.
- The supernatant was discarded and the pellets were washed with 70% cold ethanol.
- Finally the pellets were dried and resuspended in 20µL of TE buffer and stored at -20° C for further use.

The DNA extracted by the above procedure was contaminated with RNA, so the extracted DNA were treated with RNase to digest the RNA present in the preparations.

3.10 RNase Treatment of DNA Sample

In the process of RNase treatment, 10mg /mL RNaseA (Sigma Cat # R-4875) were added in distilled water and boiled for 20 minutes to dissolve. The solution was then cooled and stored in at -20^{0}C for further use.

The following steps are taken to purify the DNA with RNase A solution:

- In the DNA samples isolated so far, RNase A solution were added @ 50µg/mL and incubated for one

hour at 37^{0}C in dry bath(Genei, India).

- The RNaseA treated DNA samples were extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The tubes were centrifuged at 10,000rpm for 10 minutes and upper phase was taken in a fresh tube.
- Equal volume of chloroform: isoamyl alcohol (24:1) was added to the tube and mixed properly. The tubes were centrifuged at 10,000rpm for 10 minutes and upper phase was again taken in a fresh tube.
- In this tube double volume of ice cold ethanol and 100µL of Ammonium acetate was added and centrifuged for 12,000 rpm for 30 minutes at 4 °C.
- The supernatant was discarded and the pellets were washed with 70% cold ethanol.
- Finally the pellets were dried and resuspended in 20µL of 1X TE buffer and stored at -20° C for further use.

The RNase treated DNA was further purified with PEG, glass milk and Sigma kit to eliminate plant phenolics.

3.11 PEG Purification of DNA

Requisite amount of PEG8000 was dissolved in 2.5M aqueous solution of NaCl.

The following steps are taken to purify the DNA with PEG 8000 (Polyethylinglycol 8000, Hi-Media, Cat no #RM7402):

- DNA samples were taken and make the volume up to 50 µL by adding fresh TE buffer.
- In this tube 50 µL of 20% PEG solution was added and mixed properly.
- The mixture was incubated at 37^{0}C for 15 minutes.
- The DNA-PEG mixture was centrifuged at 17,000rpm for 15 minutes at room temperature.
- The supernatant was discarded carefully.
- In this tube 125 µL of ice cold 80% ethanol was carefully added at the wall of the tube and washed twice.
- Ethanol was dried off completely.
- The DNA was resuspended in 25 µL of fresh 1X TE buffer and stored at -20° C for further use.

3.12 Purification of DNA By Glass Milk

- In 1L glass beaker, 400g of borosilicated glass powder was resuspended in 800mL double distilled water.
- The preparation was stirred for 1 hour.

- Stir plate was then turn off and allowed the slurry to settle for 90 minutes.
- The supernatant was removed, which contained the fine glass particles, which can be used for glass milk, and put into a 250mL centrifuge bottle.
- The glass particles were pelleted by spinning at 6000rpm for 10 minutes.
- The pellet was resuspended in 250mL double distilled water.
- Concentrated nitric acid was added to the glass particles suspension and make it 50%.
- The solution was stirred and heated gently and turns on the heat to "high".
- The temperature was brought near the boiling point of the slurry and then turn off the heat.
- The slurry was brought back to room temperature with continuous stirring.
- The glass particles were pelleted as in step 5.
- The glass particles were resuspended in 250mL of double distilled water.
- The glass particles were pelleted by spinning at 6000rpm for 10 minutes.
- Continue washing and spinning of glass particles was continued and spinning as above until the pH of the slurry was neutral.
- After slurry was neutralized, the glass particles were finally pelleted by spinning at 6000rpm for 10 minutes.
- The glass pellet was resuspended in double distilled water to make 50% slurry based on volume.
- The glass milk solution was stored at room temperature.
- DNA solution was taken in a in a 1.5mL microcentrifuge tube.
- Glass milk solution was vortexed to resuspend.
- In a tube 1µL glass milk solution was added per 1µL DNA and mixed well and 3 volume of saturated Sodium iodite solution was added.
- The mixture was incubate for 5 minutes at room temperature and mixed occasionally to keep the glass milk in suspension.
- The tubes containing the solution were centrifuged at full speed for 5 seconds.
- Pellets were washed for 3 times with 500µL wash solution and centrifuged at full speed for 5 seconds.

- The pellets were dried at room temperature for 5-10 minutes.
- To elute the DNA, glass pellet was resuspended the in an equal volume of 1X TE buffer.
- Spin tube for 30 seconds at top speed in a microfuge to pellet glass milk.
- Supernatant was carefully removed with eluted DNA into a fresh tube and stored at -20° C for further use.

3.13 Purification of DNA with Kit

The DNA was finally purified by GenElute™ PCR Clean-up Kit (GenElute™ PCR Clean-up Kit, PLN-70) provided by SIGMA, which are as follows (as per the guide line provided by SIGMA):

- A GenElute™ miniprep binding column with a blue o-ring was inserted into a collection tube. In each miniprep column, 0.5mL of the column preparation solution was added to each miniprep column and centrifuged at 12,000rpm for 30 seconds to 1 minute. Elute was discarded.
- In this tube 5 volume of binding solution was mixed with 1 volume of DNA solution and the solution mixture was transferred to the binding column. The column was centrifuged at 12,000rpm for 1 minute. The elute was discarded and the collection tube was retained.
- Binding column was replaced into the collection tube and 0.5mL of wash solution was applied in to the column and the column was centrifuged at 12,000rpm for 1 minute. The elute was discarded and the collection tube was retained.
- Binding column was replaced into the collection tube. The column was centrifuged at 12,000rpm for 2 minute, without any additional wash solution, to remove excess ethanol. The residual elute as well as collection tube was discarded.
- The column was transferred to a fresh 2mL collection tube and 50µL of elution solution or purified sterile water was added to the centre of each column. The tube was incubated at room temperature for 1 minute.
- DNA was centrifuged at 12,000rpm for 1minute. The eluate is ready for immediate use or storage at -20^{0}C.

3.14. Quantification of DNA

Quantification of DNA was done with UV vis-spectrophotometer (Thermo Electro Corporation, England). Spectrometer for DNA measurement

was set to the wave length 260 nm and 280nm.

Procedure

600µL of 1X TE buffer was taken in a clean cuvette as blank (-ve control), set zero. Six (6) µL of buffer was replaced with same amount of DNA and mixer thoroughly. Optical density (OD) was taken at both 260nm (OD_{260}) and 280 nm(OD_{280}). The ratio of OD_{260}/OD_{280} was calculated to assess the purity of DNA.

3.15 Agarose Gel Electrophoresis of Genomic DNA

The size of the isolated DNA samples were analyzed by agarose gel electrophoresis as described below:

- Agarose gel was prepared using molecular biology grade, DNase free agarose (gelling temperature 36^0C) (Sigma Cat no # A 9539). For agarose gel electrophoresis of genomic DNA as well as PCR product 0.8% gel is used.
- For 30mL gel, 0.24 gm of agarose was dissolved in 30mL of 1X TBE buffer (See appendix II for composition) by heating in microwave oven (LG make).
- The gel was cooled up to 40^0C and 0.5µg/mL Ethidium Bromide (3,8-diamino-10-ethyl-9-phenylphenaanthridinium bromide, $C_{12}H_{20}BrN_3$, MW 394.33, Sigma Cat #E 8751) was added to the gel for the staining of DNA.
- The gel was cast on a gel platform (100x70mm) (Tarsons, Cat no # 7030) keeping the well end towards the cathode side.
- The gel was submerged in tank by adding 1X TBE buffer (See appendix II for composition).
- Carefully 2µL of DNA was mixed with 3µL of loading buffer (TypeIII, Sambrook and Russell, 2001) (For composition and preparation see appendix II) and was loaded into wells.
- Lambda DNA */EcoRI/ Hind III* double digest was used as a molecular marker (Genei Cat no #MBD 3L).
- The DNA was made to run a constant volt of 60V applied with Electrophoresis Power Supply Unit (Tarsons, Cat no# 7090).
- After one hour of run, the gel was viewed on a UV Transilluminator (Genei, Cat no# SF850).

3.16 Development of A New 16s Primer Sequence for *Frankia*

DNA obtained from the root nodules of *A. nepalensis* and *C. equisetifolia* were composed of mixture of DNA from the host plant, the *Frankia* and possibly

contaminating bacteria, which might not have been eliminated during the surface sterilization and peeling of nodules. Hence *Frankia* specific primers were required for obtaining exclusive *Frankia* genome band. A new 16s specific PCR primer sequence was developed with the help of *in sillico* PCR amplification of *Frankia.* Initially primers FGPS989ac (Bosco *et al.*, 1992) 5'GGGGTCCGTAAGGGTC3' and primer FGPS1490 (Normand *et al.*, 1996) were chosen. However, the primer pair failed to amplify any of the complete *Frankia* genome sequence *in silico*. Therefore the primers have been modified suitably to make them compatible to *Alnus* and *Casuarina* isolates and modified accordingly that can amplify the reference strains ACN14a and CcI3 only. These modified primers were originally designed, synthesized commercially. The primers were as follows: forward -5'-GGGTCCGTAAGGGTC-3' and reverse 5'-AAGGAGGTGATCCAGCCGCA-3'. The software Primer3 (Rozen and Skaletsky, 2000) developed those primers from the 16s sequence of *Frankia* sp. CcI3 (NC 007777), *Frankia alni* ACN14a (NC 008278) and *Frankia* sp. EAN1pec (NC 009921) available in GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome and cmd=search andterm=*Frankia*). The efficiency of the primers is were checked with the software '*In Silico* PCR Amplification' (http://insilico.ehu.es/PCR/). (Bikandi *et al.*, 2003). A 520 bp long amplicon was expected. Annealing temperature depends upon the size and nature of the primers and was calculated with the following formula.

Annealing temperature (°C) =

{2(A+T) +4(G+C)} -5

Though the two primers (Forward and Reverse) had different annealing temperature, the lowest one (58°C) was used.

3.17 PCR Amplification of DNA

The Polymerase Chain Reaction was carried out in a 50 µL volume. The reaction mixture contains the following ingredients:-

The dNTP mixture = 0.2 µM of each dNTP.

Taq DNA polymerase = 2U per reaction.

Primers = 1 µM each.

PCR Buffer (10X) = 5 µL per reaction.

Template DNA = 1µL per reaction (< 1 µg/100 µL).

The reaction mixture was adjusted to a final volume of 50µL by adding pyrogen free water. The polymerase chain reaction was performed on a Applied Biosystem thermal cycler (model 2700). The amplification reaction was performed with a negative control. This negative control did not contain any template DNA. The amplification cycles consisted of following steps:

1. First Cycle: Denaturation at 94^{0}C for 5min, primer annealing at 58^{0}C for 2 minute and primer extension at 72^{0}C for 2 minute.

2. Cycle 2-34: Denaturation at 94^{0}C for 1min, primer annealing at 58^{0}C for 2 minute and primer extension at 72^{0}C for 2 minute.

3. Cycle 35: Denaturation at 94^{0}C for 1min, primer annealing at 58^{0}C for 2 minute and primer extension at 72^{0}C for 7 minute.

3.17.1 Electrophoresis of Amplified DNA

The PCR products were analyzed by agarose gel electrophoresis as described below:

- Agarose gel was prepared using molecular biology grade, DNase free agarose (Sigma Cat no # A 9539).
- For agarose gel electrophoresis of PCR product 0.8% gel is used.
- For 30mL gel, 0.24 gm of agarose was dissolved in 30mL of 1X TBE buffer (See appendix II for composition) by heating in microwave oven (LG make).
- The gel was cooled up to 40^{0}C and 0.5µg/mL Ethidium Bromide (2, 7-diamino-10-ethyl-9-phenylphenaanthridinium bromide, $C_{12}H_{20}BrN_3$, MW 394.33, Sigma Cat #E 8751) was added to the gel for staining of DNA.
- The gel was cast on a gel platform (100x70mm) (Tarsons, Cat no # 7030) keeping the well ends towards the cathode side. The gel was submerged in tank by adding 1X TBE buffer (See appendix II for composition).
- 10µL of DNA was mixed with 2.5µL of loading buffer (TypeIII, Sambrook and Russell, 2001) (For composition and preparation see appendix II) and was loaded into wells.
- Lambda DNA *EcoRI/ Hind III* double digest and 1kb DNA marker were used as a molecular marker (Genei Cat no #MBD 3L).
- The DNA was made to run at constant volt of 60V applied with Electrophoresis Power Supply Unit (Tarsons, Cat no# 7090)

- After two hour of run the gel was viewed on a UV Transilluminator (Genei, Cat no# SF850).
- The size of the PCR product was determined with UltraLum Gel documentation software.

3.18 Purification of PCP Product

The PCR products were further purified by GenElute™ PCR Clean-up Kit (GenElute™ PCR Clean-up Kit, PLN-70p) rovided by SIGMA as per the guide line provided by SIGMA, which are as follows:

- A GenElute miniprep Binding column with a blue o-ring was inserted into a collection tube. 0.5mL of the column preparation solution was added to each miniprep column and centrifuge at 12,000rpm for 30 seconds to 1 minute. Elute was discarded.
- Five volumes of Binding solution was mixed with 1 volume of PCR reaction mix and the solution was transferred to the binding column.
- The column was centrifuged at 12,000rpm for 1 minute.
- Elute was discarded and the collection tube was retained.
- Binding column was replaced into the collection tube. 0.5mL of diluted wash solution was applied in to the column and the column was centrifuged at 12,000rpm for 1 minute. The elute was discarded and the collection tube was retained.
- The column was again centrifuged at 12,000rpm for 2 minute, without any additional wash solution, to remove excess ethanol. The residual elute as well as collection tube was discarded.
- The column was transferred to a fresh 2mL collection tube. 50µL of elution solution or water to the centre of each column. Incubate at room temperature for 1 minute.
- DNA was centrifuged at 12,000rpm for 1minute. The elute was ready for use or stored at -20^{0}C.

3.19 Digestion of PCR Products

- In a sterile microcentrifuge tube 10µL of PCR amplified product was taken.
- In this tube 2µL of 10X restriction enzyme digestion buffer (supplied with Bangalore Genei Pvt Ltd restriction enzyme) was added and mixed thoroughly.
- The PCR products were subjected to 5U of restriction digestion with various restriction enzymes, which includes *Alu*I, *Taq* I, *Hae* III, *Mbo*I and *Msp*I (Bangalore Genei Pvt Ltd) (Table 3.1).

Table 3.1: List of Restriction enzymes used in RFLP study

Restriction Enzymes with Cat# (Bangalore Genei, India)	Sequence	Reaction Volume	Reaction Temperature (°C)	Amount of RE used
Alu I (MBE -17-S)	AG↓CT	20µl	37	5U
*Taq*I (MBE -7S)	T↓CGA	20µl	65	5U
*Hae*III (MBE -10S)	GG↓CC	20µl	37	5U
*Mbo*I (MBE -27S)	↓GATC	20µl	37	5U
*Msp*I (MBE -31S)	C↓CGG	20µl	37	5U

- The tubes were incubated in a dry bath at indicated reaction temperature for 1 hour.
- The reaction was stopped by adding 0.5 M EDTA (pH 8.0) to a final concentration of 10mM. The digested DNA was stored at -20°C for further use.

3.19.1 Analysis of Restriction Fragments

RFLP analysis was done performing the gel electrophoresis of the digested product. A 2 %(w/v) molecular biology grade high resolution agarose (Sigma Cat no # A 9539) gel was prepared in 1X TBE buffer for a 15x10 cm casting tray. Electrophoresis was done in mini submarine electrophoresis unit (Tarson Cat # 7050). 20 µL of digested DNA was electrophoresed in 3V/cm electric field. Minisize 50bp DNA ladder (Norgen Biotech, Canada, Cat # 11200) was used as molecular weight standards. The gel was viewed on a UV Transilluminator (Bangalore Genei, Cat no# SF850) and photographed using Kodak digital science DC 120 digital camera.

3.19.2 Scoring of data

The PCR RFLP data was scored as present (1) or absent (0) using the SIMQUAL program and DICE coefficients. A matrix was computed and dendograme was developed by a UPGMA clustering of the POPGENE to calculate the diversity among the *Frankia* strains by Nei genetic diversity (Nei, 1987), Shannon index (Shannon, 1948; Weaver and Shannon, 1949).

Chapter 4
RESULTS & DISCUSSION

4.1. Isolation of The Bacterium

The cortical layers of actinorhizal root nodules are heavily contaminated with soil microorganisms. The growth rate of which is generally faster than the *Frankia*. So, the prerequisite for successful isolation of *Frankia* was to eliminate these contaminants without damaging the endophyte in the cortical tissue. Of the three procedure tried to isolate the endophyte, procedure III (Bose and Sen, 2006) was found as the most suitable for easy isolation of *Frankia.* In this procedure a series of mild and easily available surface sterilizing agents were used to eliminate these contaminants, which was followed by incubation in rich Qmod (Lalonde and Calvert, 1979) medium for 48 hr at 30°C temperature with 3% activated charcoal. The surface sterilizing agents used were useful against the bacterial and fungal contaminant and the vegetative bacterial and fungal cells could be killed at high rate but the chemical resistant bacterial spores might withstand this treatment and they might germinate later on in culture medium. When the nodule lobes were incubated with rich Qmod (Lalonde and Calvert, 1979) medium for 48 hr at 30°C temperature the spores might germinate and the nascent vegetative cells might be killed by subsequent chemical treatment (Bose and Sen, 2006).

Another problem in *Frankia* isolation was the presence of plant phenolics which leached out in the medium from the piece of nodule during isolation. The plant phenolics were potential inhibitor of the growth of *Frankia.* The activated charcoal minimized the effect of phenolics by absorbing it and thus increased the efficiency of *Frankia* isolation.

The advantage of using Qmod (Lalonde and Calvert, 1979) in the post isolation maintenance of the bacterium was its nutritional richness, in which any contamination can not remain in dormant condition. Thus, Qmod (Lalonde and Calvert, 1979) medium visibly ensure an absolute contamination free condition for pure culture. Define propionate minimal medium (DPM) (Baker and O'keefe,1984) is also a very good medium for maintenance of *Frankia* cultures but this medium have some disadvantages in isolation of the endophytes. The absence of nitrogen in the DPM (Baker and O'keefe,1984) helped selectively in the growth of *Frankia* while the spore forming contaminants remain in the dormant condition for a long period. These contaminants might germinate latter while transferring in nutritionally rich medium.

Frankia colonies came as minute cottony white dots at the bottom of the tube after one month incubation in dark. Each colony was collected and subcultured in separate tubes containing Qmod (Lalonde and Calvert, 1979) medium. Although several concentrations of $VitB_{12}$ and $VitB_1$ were played in isolation medium but Vit B_{12} at 0.2% concentration in aqueous solution (1mL/L) and Vit B_1 at 0.5% concentration in aqueous solution (1mL/L) gave the best result.

4.2 Germination of Seeds of Actinorhizal Plants

The seeds of *Alnus nepalensis* and *Casuarina equisetifolia* need different conditions for germination. No special treatment was needed for germination of *C. equisetifolia* seeds and they were germinated on sterile distilled water in floating condition.

Generally, the seeds of *A. nepalensis* need a special kind of treatment in the form of overnight soaking in aerated water for better germination. The treated and non-treated conditions, media nutrients and contamination of fungus do not have any effect on the duration of seed germination. In all the cases the seeds germinated within 4 weeks. Contaminations were reported in mineral rich medium under treated and non-treated conditions. The percentage of germination varied with the media used (Fig: 4.1) in *A. nepalensis* seeds. Maximum germination was observed in Hoagland media for both treated and non treated seeds while minimum in ½ Murashige and Skooge medium (MS) for treated and ½ woody plant medium (WPM)

for non treated seeds. The germination was probably inhibited by high nutrient concentrations. For treated seeds, maximum (80%) survival rate was reported in Hoagland media while minimum (0%) survival rate was reported in WPM. For non-treated seeds, maximum (100%) survival rate was reported in Hoagland while minimum (20%) survival rate was reported in half strength WPM. Moderate germination and survival rate with low contamination were reported in water used as germination medium (Fig 4.2).

The hormones had profound but variable effect on the seed germination and rooting of *A. nepalensis*. IBA and NAA+IBA were found to be more effective than NAA in terms of germination percent and rooting. Moderate germination percentages (above 50%) were observed in the media supplemented with IBA 1 and 2 mg/L and NAA+IBA 1+1 and 2+2 mg/L. Maximum (90 %) germination was observed with the application of IBA (1 mg/L) and NAA + IBA (1+1 mg/L). The germination percent increased with the increase of NAA concentration while declined with the increase of IBA and NAA + IBA concentrations. Good rooting and good growth of roots were observed in the media supplemented with IBA 3, 4 and 5mg/L and NAA + IBA (1+1 mg/L). The best result was recorded in the medium supplemented with 4mg/L IBA. In the medium supplemented with NAA + IBA (2+2 mg/L) rooting was poor but the roots showed good growth.

In the study of seed germination in sterilized vermicompost, good germination rate was reported within 10 days but the seedlings did not survive. This death event was due to poor root development. In double layer culture experiment both the germination percentage and survival rate were very low although the media contained required hormones as described in earlier experiments.

These experiments reveals that germination of *A. nepalensis* seedlings have specific hormonal requirements. These hormonal requirements are fulfilled by mycorrhizal or associative fungi during germination and growth in nature. In vermicompost the *Alnus* seedlings did not survive though there were plenty of nutrients. The nutrients were not absorbed due to poor root development which require external hormonal supply. This plant *A. nepalensis*, thus begins its life with an association with fungi and at its

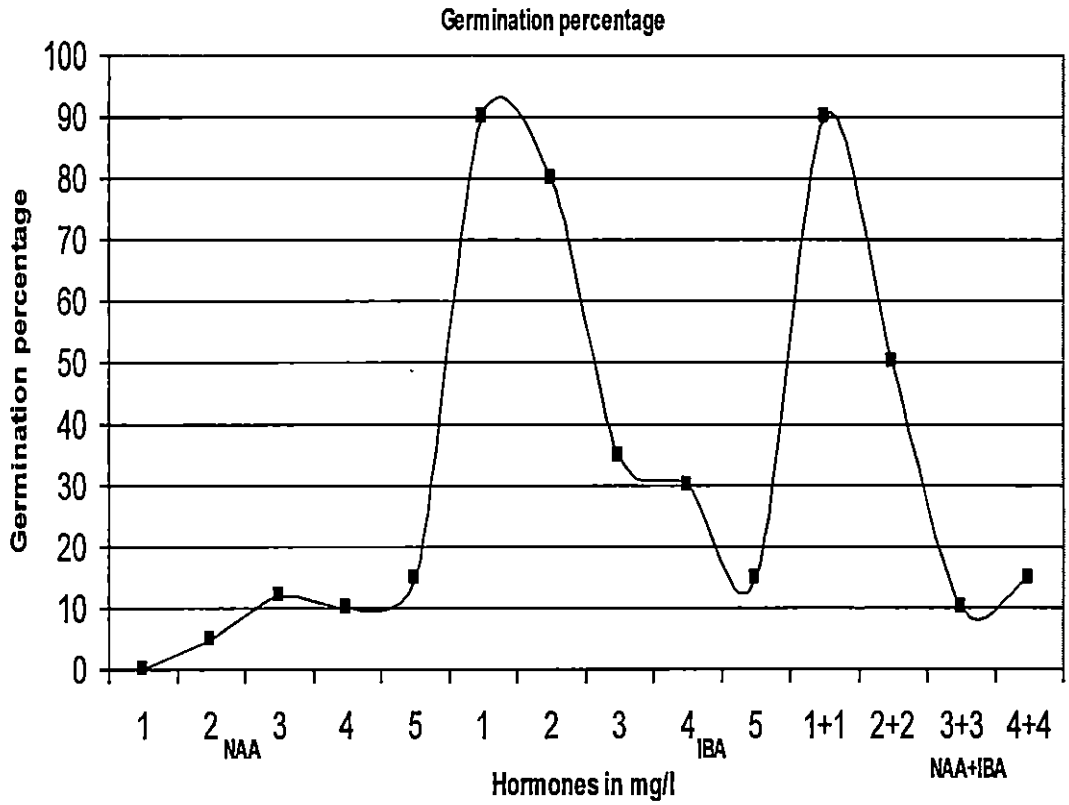


Fig 4.1: Effect of various hormone concentrations on *A. nepalensis* seed germination percentage.

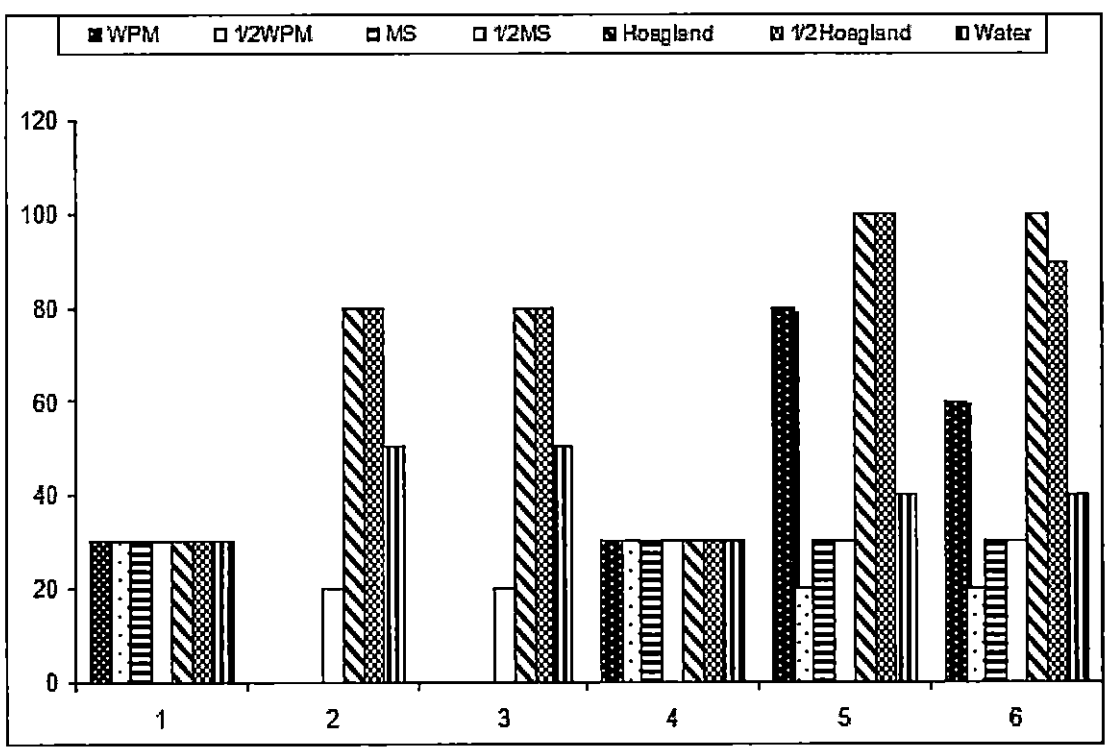


Fig 4.2: Generation time (G time), Generation percentage and survival rate of treated and untreated *A. nepalensis* seeds in different medium.

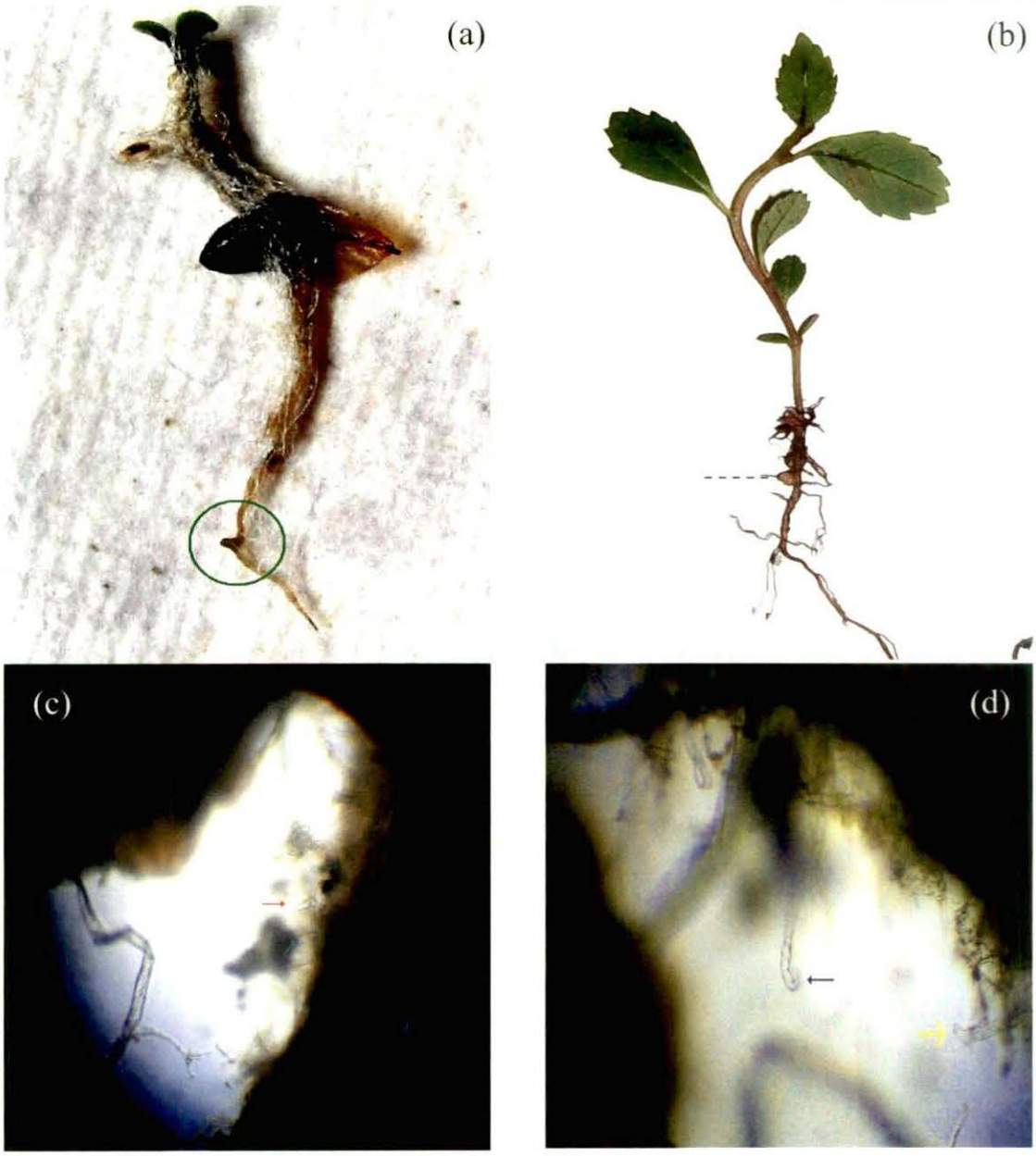


Fig 4.3: a) A young *C. equisetifolia* seedling with nodule; b) A young *A. nepalensis* seedling with nodule; c) A young root hairs show ignition of root hair deformation due to *Frankia* infection: d) A young root hairs show typical hook shaped root hair deformation due to *Frankia* infection.

maturity make another association for survival with an actinomycetes bacteria *Frankia*. *C. equisetifolia,* did not have this problem.

4.3 Plant Infectivity Test

The characteristic root hair deformation and visible nodule formation (Fig 4.3) were observed both in *A. nepalensis* and *C. equisetifolia* seedlings grow in aseptic condition. This indicates the presence of *Frankia* in the medium.

4.4 Colony Morphology

Medium used: Defined propionate minimal medium (Baker and O'keefe, 1984)

Reaction pH: 7

A. Size: Filamentous.

Temperature: 28^{0}C. **Age:** 30 days

B. Motility : Not observed

C. Sporangia and spores: Present, sporangia multilocular, spore round shaped smooth walled in older culture. 2mM Pb increase sporulation.

D. Agar colonies :

Temperature : 28^{0}C

Age : 14 days.

Location : Deep colony

Form : Spindle shaped

Edge : Filamentous

Optical character: Opaque

Pigmentation : Red in 2mM Pb concentration.

E. Broth:

Temperature : 28^{0}C

Age : 14 days.

Odor : None

Clouding : Absent

Surface growth: Absent

Sediments : Flanky

Amount of sedimentation: Scanty

F. Forms and arrangement: Filamentous

G. Gram staining : Positive

H. Biological relationship: Symbiotic to actinorhizal plants.

I. Isolated From : DPM (Baker and O'keefe, 1984) lead plates containing 2mM Lead. (Bose and Sen, 2005)

4.5 Field Emission Scanning Electron Microscopy

The hyphae, sporangium, and vesicles are the most important structures of *Frankia*. The multilocular sporangium, round shaped vesicle structures were found. The structure of sporangium was ±1.5 x 0.7 µ. The mean diameter of the hyphae was ± 0.5µ. The mean diameter of vesicle was ± 0.7µ. These results were nicely fitted with a classical frankial structure (Fig 4.4a & 4.4b).

4.6 Biochemical Characters of *Frankia*

Eleven *Frankia* strains were selected and used in the study of physiology of the bacterium. Out of these eleven strains six were isolated from *C. equisetifolia* and the rest from *A. nepalensis.*

The three *Frankia* strains (CeSi10, CeSi11, CeSi12) isolated from *C. equisetifolia* showed rapid decarboxylation activity with propionate and pyruvate and very slow decarboxylation activity with acetate and citrate. Decarboxylation leads to a rise in pH assessed as change in colour of the indicator from yellow - orange to red. These strains showed high protease activity which were demonstrated by the liquefaction of gelatin. None of these strains showed β

-glucosidase activity. They could utilize a variety of organic acids and a few carbohydrates. They had utilized propionate, acetate, succinate, sucrose and fructose as sole carbon source as they produce visible colonies within 60 days of incubation, but they can not utilize lactose, glucose, pyruvate, mannitol, tween80 and citrate.

The other three *Frankia* strains (CeSt2, CeSt5, CeSt9) isolated from *C. equisetifolia* showed no decarboxylation activity with propionate, pyruvate, acetate and citrate. None of these strains showed β-glucosidase activity, though they showed very slow protease activity. They could utilize propionate, acetate and succinate as sole source of carbon but they could not utilize glucose, pyruvate, mannitol, tween 80, citrate, sucrose, fructose and lactose. All the above experiments were done in triplicate and same results were found . The results are summarized in table 4.1 and table 4.2.

It was very interesting to note that these *Frankia* strains could not utilize the tween compounds which is a very common carbon source for *Frankia.* But non utilization of tween compound is not uncommon in *Frankia* strains. Several well recognized strains could not recognize tween compounds which include $ACN1^{AG}$, EAN1pec, and EuIIc etc. A possible explanation of the difference between the two groups of *Frankia* is that they lack, an active carbohydrate transport system (Prof. Normand, personal communication).

From the above result it could be concluded that the three strains namely CeSi10, CeSi11 and CeSi12 belongs to the physiological group 'A' of the *Frankia* which is more saprophytic than symbiotic while the other strains namely CeSt2, CeSt5, CeSt9 belongs to the physiological group 'B' of the *Frankia* which are physiologically less active and more symbiotic. Overall the strains utilize the propionate and acetate as best carbon source. So in this area results showed diversity among the *Frankia* on the basis of standard physiological parameters. These tests can be a useful addition to the methods used in differentiating *Frankia* strains isolated from *C. equisetifolia.*

Similar physiological experiments were also performed with the *Frankia* strains isolated from *A. nepalensis.* Two *Frankia* strains (AnMr1, AnMr 5) isolated from the root nodules of *A. nepalensis* growing in Darjeeling Hills showed rapid decarboxylation activity with propionate and pyruvate and

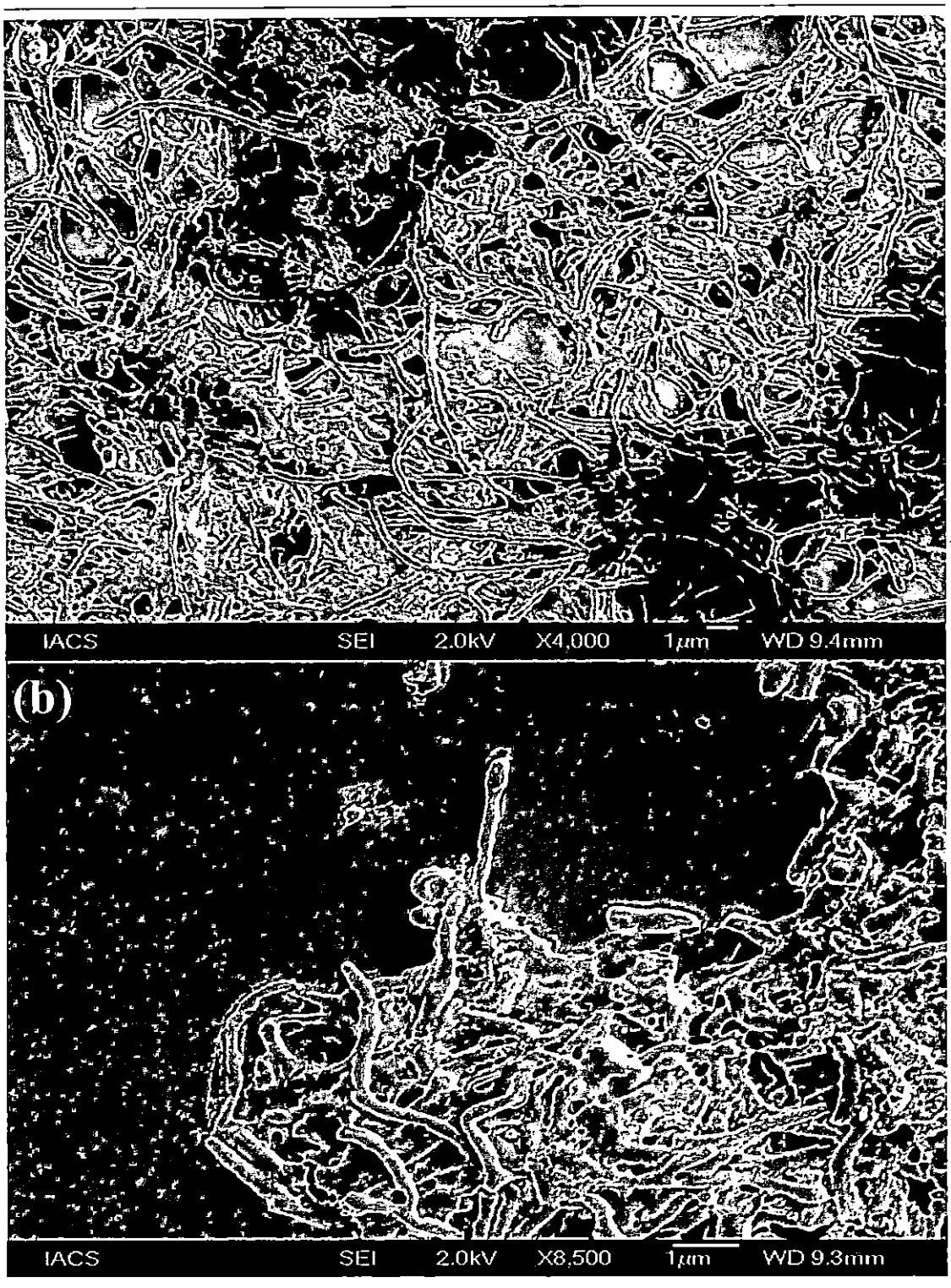


Fig 4.4: a) FESEM picture of multilocular sporangia of *Frankia;* b) FESEM picture of vesicle of *Frankia*

another three *Frankia* strains (AnMr2, AnMr2a AnMr2b) isolated from the root nodules of the same plants also growing in the same area showed no decarboxylation activity with propionate and pyruvate and the strains AnMr2b showed slow decarboxylation activity with acetate and citrate as

Table 4.1: Utilization of different carbon source by *Frankia* isolated from *A. nepalensis* and *C. equisetifolia.*

	Alnus specific strains				
	AnMr1	AnMr5	AnMr2	AnMr2b	AnMr2a
Propionate	+	+	+	+	+
Pyruvate	+	+	+	+	+
Acetate	+	+	+	+	+
Succinate	+	+	+	+	+
Glucose	(-)	(-)	(-)	(-)	(-)
Sucrose	(-)	(-)	(-)	(-)	(-)
Lactose	(-)	(-)	(-)	(-)	(-)
Fructose	(-)	(-)	(-)	(-)	(-)
Tween 80	+	+	+	+	+
Glucose + Tween 80	(-)	(-)	(-)	(-)	(-)
Mannitol	+	+	+	+	+
Citrate	+	+	+	+	+

	Casuarina specific *Frankia* strains					
	CeSi 10	CeSi 11	CeSi 12	CeSt 2	CeSt 5	CeSt 9
Propionate	+	+	+	+	+	+
Pyruvate	(-)	(-)	(-)	(-)	(-)	(-)
Acetate	+	+	+	+	+	+
Succinate	+	+	+	+	+	+
Glucose	(-)	(-)	(-)	(-)	(-)	(-)
Sucrose	+	+	+	(-)	(-)	(-)
Lactose	(-)	(-)	(-)	(-)	(-)	(-)
Fructose	+	+	+	(-)	(-)	(-)
Tween 80	(-)	(-)	(-)	(-)	(-)	(-)
Glucose + Tween 80	(-)	(-)	(-)	(-)	(-)	(-)
Mannitol	(-)	(-)	(-)	(-)	(-)	(-)
Citrate	(-)	(-)	(-)	(-)	(-)	(-)

+ = Visible colony occur within 30 days of incubation; (-) = No visible colony occur within 30 days of incubation;

shown earlier by *Frankia* isolated from *C. equisetifolia.* The AnMr1 and AnMr5 show high protease activity which is demonstrated by the liquefaction of gelatin while rest of the strains shows very slow protease activity. None of these strains showed β-glucosidase activity. These strains could utilize a variety of organic acids and a few carbohydrates. They utilized propionate, acetate, succinate, pyruvate, mannitol, tween80 and citrate as sole carbon source as they produce visible colonies within 60 days of

Table 4.2. Physiological characteristics of *Frankia* isolated from *A. nepalensis* and *C. equisetifolia*

	ACN1[AG*]	AnMr1	AnMr5	AnMr2b	AnMr2	AnMr2a	CeSi10	CeSi11	CeSi12	CeSt2	CeSt5	CeSt9
Dec. of acetate	(-)	+	+	+	+	+	+	+	+	(-)	(-)	(-)
Dec. of propionate	(-)	++	++	(-)	(-)	(-)	++	++	++	(-)	(-)	(-)
Dec. of citrate	(-)	+	+	+	+	+	+	+	+	(-)	(-)	(-)
Dec. of pyruvate	(-)	++	++	(-)	(-)	(-)	++	++	++	(-)	(-)	(-)
Protease activity	++	++	++	(-)	(-)	(-)	++	++	++	(+)	(+)	(+)
β-glucosidase activity	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)

Dec. = Decarboxylation; + = Positive result (with in two to three weeks); ++ = High activity (positive result with in two weeks); (+) = Very slow (positive result with in four weeks); (-) = Negative result with in 42 days of incubation; *= Reference strain.

incubation, but they could not utilize lactose, glucose, fructose and sucrose. All the above experiments were done in triplicate and same results were found. The results are summarized in table 4.1 and table 4.2.

From the above result it could be concluded that the strains isolated from the Darjeeling Hills belong to the physiological group 'A' of the *Frankia* which were more saprophytic than symbiotic. Overall the strains utilize the propionate and acetate as best carbon source. So, in this area we were able to detect the diversity among the *Frankia* on the basis of standard physiological parameters. These tests could be a useful addition to the methods used in differentiating *Frankia* strains isolated from *C. equisetifolia* and *A. nepalensis*.

4.7 Statistical Analysis of The Biochemical Data by Software POPGENE

The result of these physiological tests were analyzed by POPGENE and the diversity of *Frankia* were determined by Nei genetic diversity (Nei, 1987), Shannon index (Shannon, 1948; Weaver and Shannon, 1949), single locus component and two locus component (Brown and Feldman,

1981) and Wahlund effect (Brown and Feldman, 1981). Among the *Frankia* population the total genetic diversity had been found to be 0.281 (SD ± 0.0547), which showed low level of genetic diversity. The estimated gene flow within the population was zero. The coefficient of gene differentiation among population indicated what percentage of total genetic diversity came from interpopulation and in this case, there was no lateral gene transfer in *Frankia* strains. So, the entire genetic diversity came from intrapopulation source and hence the value of coefficient of gene differentiation was 1.

Shannon index is used to study the diversity of the population. It is assumed that all species are represented in the sample and they are randomly sampled. The advantage of this index is that it takes into the account the number of species and the evenness of the species. The index is increased either by having additional unique species or by having greater species evenness. The value of Shannon index will be maximum if the evenness present in the population is maximum. It has specific importance in the expression of equal proportion character in the sample rather than the total number of individuals. The Shannon index has moderate sensitivity towards the sample size. In the present study the value of Shannon index had been found as 0.3982 (SD= ± 0.3293), which indicated a moderate level of diversity.

Another important diversity measurement index used in this study was the single locus component and and two locus component. The single locus components are the average and the variation among the population in gene diversity and variance among the population in allel frequency. The two locus component include the mean and variance of disequilibria, the covariance of allel frequencies, over population and various interactions. Wahlund effect is used to explain the single locus component and and two locus component. It refers to the reduction in the heterozygocity in the population caused by subpopulation structure. The *Frankia* population showed Wahlund effect 2.7071 for single locus effect and 8.901 for the two locus effect. If the subpopulations have the same gene frequency the value of Wahlund effect will be zero. In this case the value represented very low level of genetic diversity among *Frankia* population.

The dendrogram Based on Nei's (1987) Genetic distance (Method = UPGMA Modified from NEIGHBOR procedure of PHYLIP Version 3.5) (Fig 4.5) showed that the *Frankia* population was divided in to two major clades. The first clade included the bacterium isolated from *C. equisetifolia* and the second clade included bacterium isolated from *A. nepalensis*. Each clade was again sub divided into two minor clades. This pattern of the dendrogram supported the presence of two physiological groups among the bacteria isolated from each host.

Thus from the result of Nei genetic diversity (Nei, 1987), Shannon index (Shannon, 1948; Weaver and Shannon, 1949), single locus component and two locus component (Brown and Feldman, 1981) and Wahlund effect (Brown and Feldman, 1981), it can be concluded that that the *Frankia* population present in this area shows very low genetic diversity and the total genetic diversity was intrapopulation in origin and divided into two different physiological group (Fig4.5). (Bose *et al.*, 2011)

4.8 Heavy Metal Salt Resistance Pattern

Heavy metal salt resistant colonies of *Frankia* appeared after around 14 days of incubation. The numbers of colonies were plotted against the increasing concentration of various heavy metal salts (Fig 4.6; 4.7) and the minimum inhibition concentrations (MIC) calculated (Table 4.3). Figure 4.6 shows the growth pattern of *Frankia* strains isolated from *C. equisetifolia* in presence of different metal salts. The results show that the strain was highly resistant to cadmium chloride (5mM), moderately resistant to cobalt chloride and lead nitrate (3mM) and less resistant to nickel chloride and copper sulphate (0.5 mM). This results were compared with the American strains and found that the present strain was much more tolerant to cadmium chloride and cobalt chloride. While the MIC value of USA strains CcI3 and Cc117 for $CdCl_2$ was 0.1 mM and for $CoCl_2$ 0.1 and 0.05 mM respectively. This strain showed a MIC value of 5 and 3 mM for $CdCl_2$ and $CoCl_2$ respectively. However, USA strains grew at an elevated concentration of $CuSO_4$, $NiCl_2$ and Pb $(NO_3)_2$ than the studied strains (Richard *et al.*, 2002). The heavy metal resistant *Frankia* colonies were re-isolated from various plates and preserved for further studies. Each experiments were repeated twice and were found to produce similar results. The strain used in the study

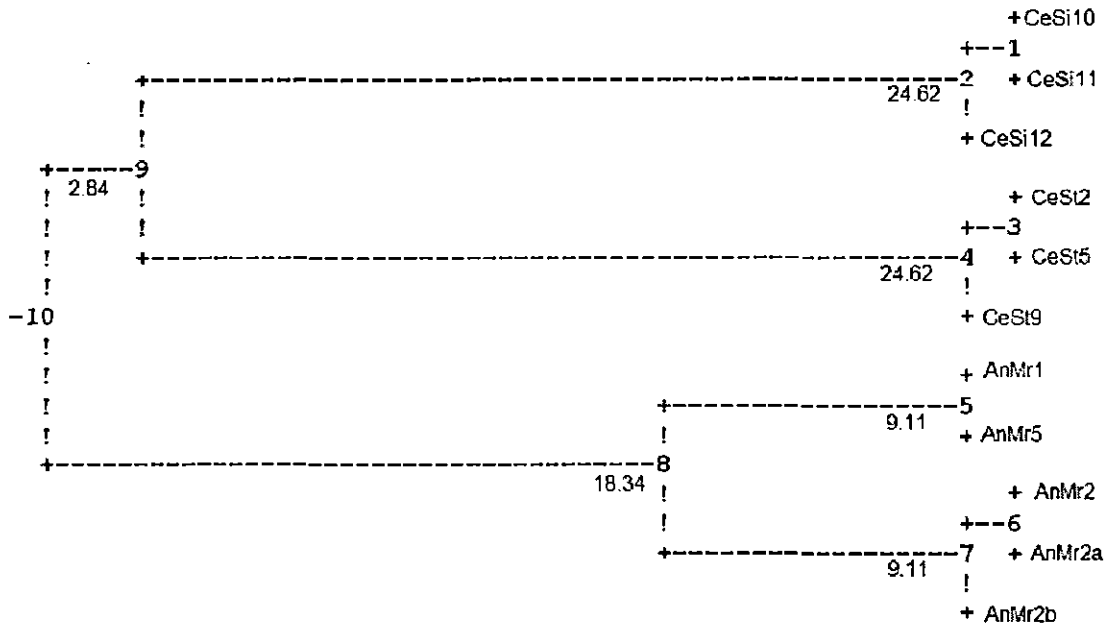


Fig 4.5: Dendrogram Based on Nei's (1987) Genetic distance showing the diversity of *Frankia* based on physiological results: Method = UPGMA Modified from NEIGHBOR procedure of PHYLIP Version 3.5. Numbers indicated in the bottom of the lines are root distance.

were found compatible with elevated concentration of cadmium, cobalt and lead salts.

Similarly, the same protocol was used for the heavy metal resistance pattern study in *Frankia* isolated from the *A. nepalensis*. Heavy metal salt resistant colonies of *Frankia* isolated from the *A. nepalensis* appeared similarly after around 14 days of incubation. The numbers of colonies were counted against the increasing concentration of various heavy metal salts and plotted in the graph. Fig 4.7 shows the growth pattern of *Frankia* strain AnMr1 in presence of different metal salts. The results show that the strains were highly resistant to cadmium chloride (2mM) and lead nitrate (5mM) moderately resistant to cobalt chloride (0.75mM) and less resistant to nickel chloride(0.05mM) and copper sulphate (0.5 mM). This results were also compared with the American strains (Richards *et al.*, 2002) and found that the present strains were much more tolerant to cadmium chloride and copper sulphate. While the MIC value of USA strains for $CdCl_2$ was 0.5 mM and for $CoCl_2$ 0.25, the strain, studied strains showed a MIC value of 2 and 0.5 mM for $CdCl_2$ and $CoCl_2$ respectively. However, USA strains grew at an elevated concentration of $NiCl_2$ and $Pb(NO_3)_2$ than the studied strain. The heavy metal resistant

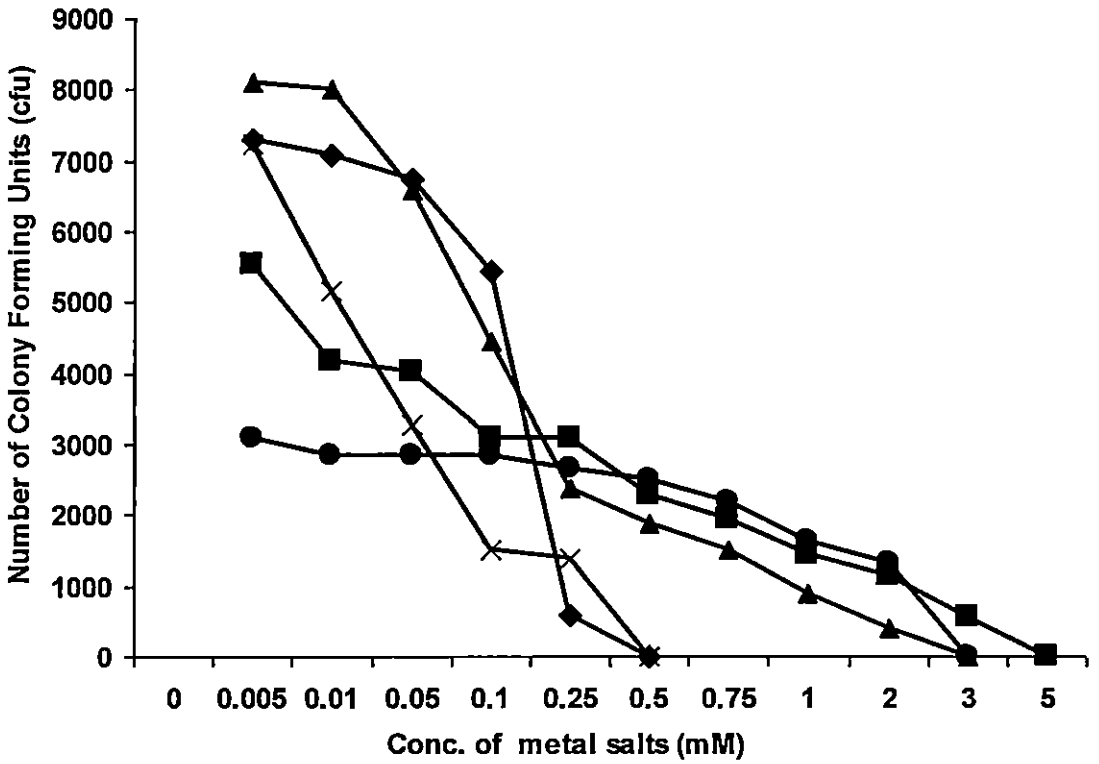


Fig 4.6. Effect of different heavy metal salts on growth of *Frankia* strain (CeSi5) isolated from *C. equisetifolia;* ; ¬ ♦- nickel chloride, ●-cadmium chloride, ▲-cobalt chloride, ■-lead nitrate and X-copper sulphate (Bose and Sen, 2006).

Frankia colonies were re-isolated from various plates and preserved for further studies. Each experiment was repeated twice and was found to produce similar results.

A characteristic heavy metal induced either purple or red or both type of pigment production was noted in all the cases except in lead at MIC or higher concentrations. This type of pigment production is totally different from the pigment production of the strains isolated from *C. equisetifolia* growing in North Bengal University campus (Bose and Sen, 2005, 2006) where a faint red pigment was produced only in lead plates.

When these two results were compared it was found that *Frankia* strain isolated from *A. nepalensis* were less resistant to the heavy metal salts. It was hypothesized that the *Frankia* isolated from *A. nepalensis* facing less heavy metal stress than *Frankia* isolated from *C. equisetifolia.* As the latter was the inhabitant of coastal region which was facing the more metal concentrations due to metal pollution and become more resistant to heavy metal ions. On the other hand hilly forest region of the Darjeeling district is relatively free from metal pollution and hence native *Frankia* were less exposed to heavy metal ions. One of the strain AnMr1

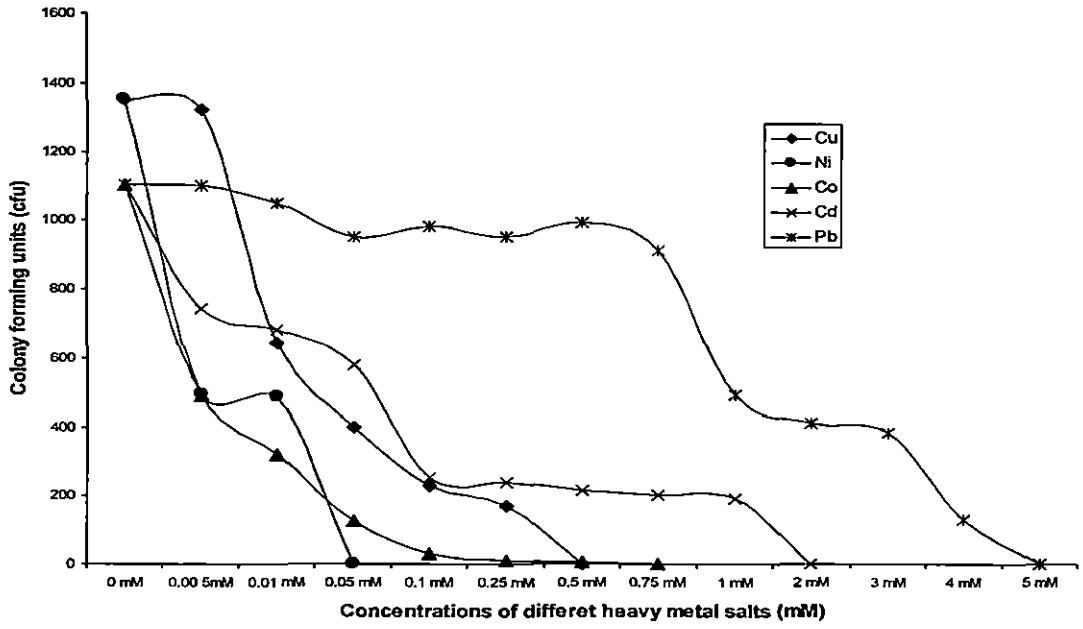


Fig 4.7: Heavy metal resistance pattern of *Frankia* strain (AnMr1) isolated from *A. nepalensis*. (Bose and Sen, 2007).

was found compatible with elevated concentration of cadmium and lead salts.

This had great significance since the heavy metal resistance is a more suitable marker for slow growing bacteria like *Frankia* as it is more stable than antibiotics resistance (Richards *et al.*, 2002).

4.9 Analysis of Heavy Metal Resistance Genes of *Frankia*

The heavy metal resistant genes of *Frankia* are listed in the Tab 4.4. Figure 4.8 showes the dendrogram obtained by the multiple sequence alignment of the studied genes of the three strains of *Frankia*. It was seen from the dendrogram that there was a single major clade and two subclades. The tellurite and tellurium resistant genes clustered together and the copper genes clustered together with the exception of one copper resistance gene from *Frankia* CcI3. Clustering of copper resistance and tellurite resistance genes of the different *Frankia* strains suggests that they have co-evolved as a unit. The Zn-Co-Cad resistance gene had a completely different origin and did not lie in a particular clade. (Bose *et al.*, 2007).

The heterogenecity of the codon usage was significantly related to the translation of that particular gene. The highly expressed genes, like ribosomal protein genes some have high degree of codon bias, depending on their effective number of codon (Nc)

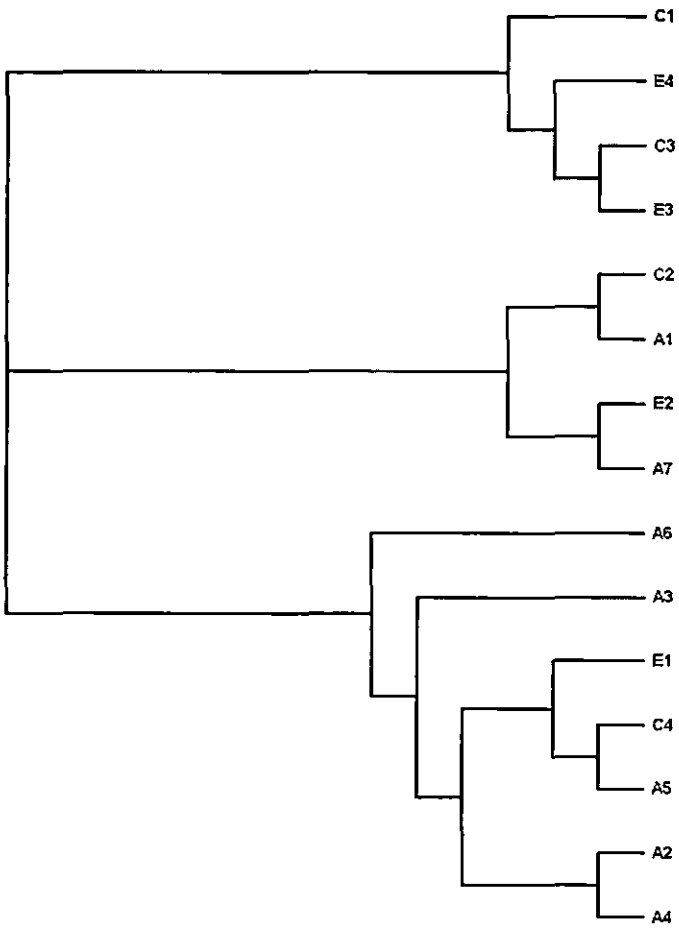


Fig 4.8: Dendrogram of the relationship of heavy metal resistance genes of the *Frankia* strains.

(Ikemura 1981; Lafay *et al.*, 2000). Thus Nc was plotted against the GC3 to investigate the diversity of codon usages in the genomes (Peden, 1999). It is a common fact that high GC containing organism show considerable codon usages diversity (Wu *et al.*, 2005). The ribosomal protein genes were considered as a reference in this work. These proteins were clustered at the lower end of the graph which was an indication of strong codon bias. The heavy metal resistance genes also clustered along the ribosomal protein genes and a strong codon bias was indicated in these cases. The *Frankia* genome showed low value of effective number of codon (<40).

The values of the codon usage indices calculated for the studied strains are shown in Table 4.4. From the results it was seen that the G+C content of the *Frankia* strains were high. Due to this property, it was hoped that the GC3 content and the Nc values for the genes from *Frankia* genomes might have some degree of heterogeneity. It was seen that the Nc values decrease with increase in GC3 content indicating that codon bias increases with the increase in GC3 content. The Nc/GC3 plot demonstrated an effective technique for

Tab 4.3: MIC and MTC of various heavy metal salts

	AnMr1					CeSi5				
Heavy metals used	Copper	Nickel	Cobalt	Cadmium	Lead	Copper	Nickel	Cobalt	Cadmium	Lead
MIC	0.01	0.005	0.005	0.005	1	0.05	0.05	3	5	3
MTC	<0.01	<0.005	<0.005	<0.005	<0.75	<0.05	<0.05	<3.00	<5.00	<3.00
Pigment production	Red	Red and purple	Red	Red	Absent	Absent	Absent	Absent	Absent	Reddish white

investigating codon usage variations among the genes. The Nc value of the genes showed a range for all the genomes suggesting that these highly GC rich genomes exhibited considerable amount of heterogeneity in codon usage. Genes coding ribosomal proteins that are known to be highly expressed were highlighted in the NC/GC3 plots. Compared to the ribosomal protein genes the heavy metal resistance genes in the genomes showed some difference. Heavy metal resistance genes of CcI3 were less biased compared to the ACN14a and EAN1pec strains. Most of the ribosomal protein genes for all the *Frankia* genomes were found to be clustered at lower ends of the plot suggesting a strong codon bias for these genes. However, heavy metal resistance genes of CcI3, ACN14a and EAN1pec strains were clustered at lower ends of the plot (Fig 4.9A,B,C). Genes with low Nc (value <40) have much stronger codon bias than be simply explained in terms of mutational bias. Ribosomal protein genes and those associated with metal resistance had a lower mean Nc value than that obtained for all of the protein coding genes suggesting a higher degree of bias in the former. These

values indicate a strong codon bias over mutational bias. This codon bias arise due to natural selection for translational efficiency (Peden, 1999)

The Codon adaptation index (CAI) assesses the extent to which selection has been effective in molding the pattern of codon usage. Heavy metal genes and ribosomal protein genes had higher mean CAI values compared to that of the protein coding genes. High CAI values indicate better expression levels. Especially the comparatively higher expression levels for heavy metal resistance indicate the ability of *Frankia* genomes to survive in stressed environments. The result obtained in this study put an additional support to the hypothesis that *Frankia* strain CcI3 is more symbiotic than saprophytic, since they remain largely in the nodules (a protective place for them) and hardly grow in the soil.

4.10 Isolation of DNA

The *Frankia* DNA was isolated using the method of Bajwa (2004). The DNA was precipitated forming DNA-CTAB complex as whitish mucus at the bottom of the microcentrifuge tubes. These precipitations were dissolved in 1X TE buffer and used for further analysis. The 0.8% agarose in agarose gel electrophoresis showed good

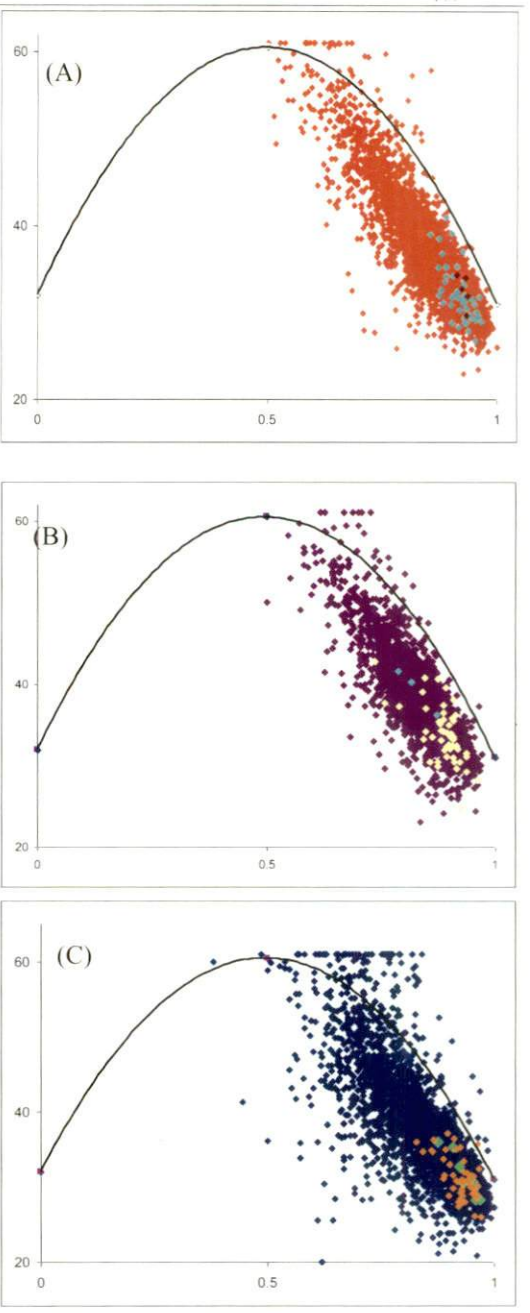


Fig 4.9 A: The NcGC3 curve of *Frankia* Strain EAN1pac: Protein coding gene: Orange; Ribosome protein gene: Green; Metal resistant genes: Pink; B : The NcGC3 curve of *Frankia* Strain CcI3: Protein coding gene: Pink; Ribosome protein gene: Yellow; Metal resistant genes: Green; C: . The NcGC3 curve of *Frankia* Strain ACN14a: Protein coding gene: Blue; Ribosome protein gene: Yellow; Metal resistant genes: Green

bands..

4.11 Purification of DNA

The genomic DNA is generally contaminated by various proteins, RNA and polysaccharides. During the DNA extraction process the proteins were eliminated by phenol and chloroform. The RNA were removed by RNaseA treatment. The polysaccharides were removed by the standard DNA extraction protocol (Bajwa, 2004). To get rid of extra phenolics which in turn hinders the downstream reactions the samples were further purified with glass milk, PEG8000 and sigma kit. These purified DNA was used for polymerase chain reaction. The purified DNA was subjected to spectrophotometric quality testing. All the samples were found to be qualitatively pure with a ratio of A_{260}/ A_{280} of ~1.8.

4.12 Development of A New 16s Primer Sequence for *Frankia*

A 20 bp long primer was developed by

Table 4.4- Values of Different Codon usages Indices for the Heavy Metal Resistance Genes of *Frankia*

Genes	Gene ID	Abbn.	CAI	CBI	Fop	Nc	GC3	GC
Copper resistance D [Ccl3]	637880116	C1	0.228	0.241	0.54	35.2	0.89	0.72
Copper resistance protein CopC [Ccl3]	637880115	C2	0.251	0.203	0.53	38.8	0.82	0.74
Tellurite resistance protein TerB [Ccl3]	637880329	C3	0.334	0.226	0.56	33.3	0.91	0.64
Putative tellurium resistance protein TerA [Ccl3]	637881001	C4	0.342	0.357	0.63	33.4	0.91	0.69
Tellurium resistance protein TerA [Ean1pec]	641239986	E1	0.332	0.369	0.63	29.7	0.94	0.71
Copper resistance protein CopC [Ean1pec]	641246078	E2	0.26	0.327	0.6	32.8	0.92	0.74
Tellurite resistance TerB [Ean1pec]	641240861	E3	0.207	0.197	0.54	36.6	0.86	0.7
Tellurite resistance TerB [Ean1pec]	641241347	E4	0.195	0.192	0.52	34.4	0.91	0.77
Putative copper resistance protein [ACN14a]	638100993	A1	0.229	0.219	0.53	38.9	0.81	0.69
Tellurium resistance protein [ACN14a]	638101739	A2	0.327	0.331	0.62	28.3	0.96	0.68
Tellurium resistance protein terE [ACN14a]	638098466	A3	0.13	0.168	0.49	49.9	0.55	0.77
Tellurium resistance protein terE [ACN14a]	638102547	A4	0.282	0.168	0.5	42.4	0.68	0.67
Tellurium resistance protein terA [ACN14a]	638102544	A5	0.246	0.165	0.49	38	0.7	0.71
Putative Cobalt-zinc-cadmium resistance protein [ACN14a]	638100325	A6	0.264	0.288	0.57	30.7	0.95	0.75
Putative Copper resistance domain [ACN14a]	638096856	A7	0.204	0.124	0.49	35.3	0.91	0.77

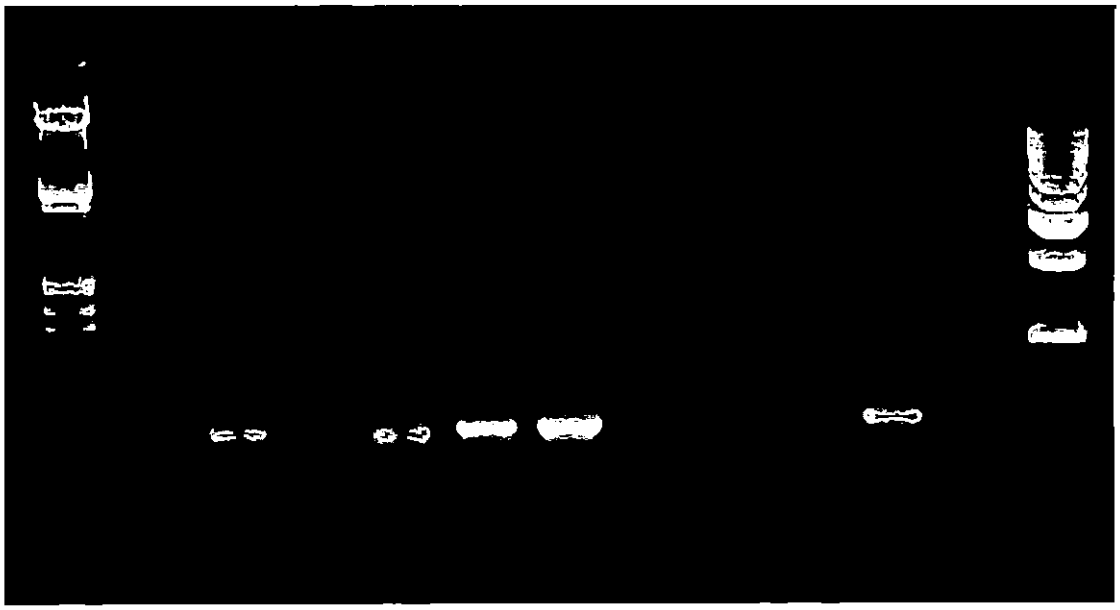


Fig 4.10 Agarose gel photograph of PCR amplification of *Frankia* DNA; Lane1, Lambda DNA *EcoRI/ Hind III* double digest DNA marker; Lane2, –ve control; Lane3-7, A1-A5; Lane8-12, C6-C10; Lane13, 1kb DNA marker (for details refer section No. 3.9.2 of MM).

Primer3. The newly designed primer set could amplify a product of 520 bp from the distal part of 16s rDNA portion of *Frankia* infecting both *A. nepalensis* and *C. equisetifolia* . The sequence was as follows:

forward-5'-GGGTCCGTAAGGGTC-3' and
reverse-5'-AAGGAGGTGATCCAGCCGCA-3'

4.13 PCR Amplification of *Frankia* DNA

The distal part of the 16s rDNA of *Frankia* was the target sequence of the newly developed primer. The primer pair then amplified a single DNA band of expected length (520bp). The PCR products were subjected to restriction digestion with various restriction enzymes, which includes *Alu*I, *Taq* I, *Hae* III, *Mbo*I and *Msp*I. Relatively low level of polymorphism was found.

4.14 Analysis of PCR-RFLP Data

The above results were analyzed with various statistical methods of POPGENE package as mentioned earlier. Among the *Frankia* population the total genetic diversity had been found to be 0.2689 (SD± 0.1858), which showed low level of genetic diversity. This low level of diversity indicates that total genetic diversity comes from inter population source and in this case, there were no lateral gene transfer occur in *Frankia* strains.

Shannon index is used to study the diversity of the population. It is assumed that all species are represented in the sample and they are randomly sampled. The advantage of this index is that it takes into account the number of species and the evenness of the species. The index is increased either by having additional unique species or by having greater species

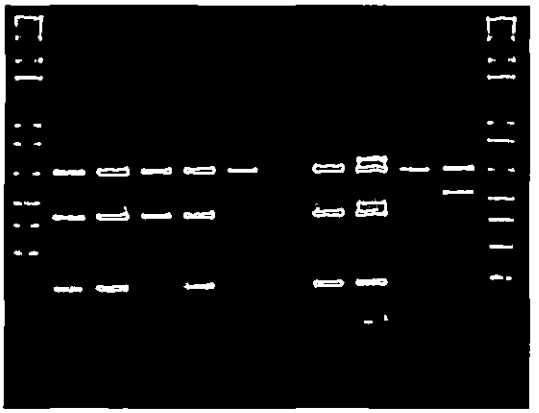


Fig 4.11a. PCR product digested with *Alu*I; Lane 1and 13- 50bp DNA ladder; Lane 2-6, A1 -A5; Lane 7-11, C1-C5. (for details refer section No. 3.9.2 of MM)

evenness. The value of Shannon index will be maximum if the evenness present in the population is maximum. It has specific importance in the expression of equal proportion character in the sample rather than the total number of individuals. The Shannon index has moderate sensitivity towards the sample size. In the present study the value of Shannon index has been found as 0.4062 (SD= ± 0.2619). This value also indicated the low level of genetic diversity.

Thus from the result of statistical analysis, it could be concluded that the *Frankia* population present in this area shows very low genetic diversity and the total genetic diversity was intra population in origin.

The dendrogram based on Nei's (1972) Genetic distance (Method = UPGMA Modified from NEIGHBOR procedure of PHYLIP Version 3.5) (Fig 4.12) showed that the *Frankia* population of *Alnus* based and *Casuarina* based were divided into three major clades. In the first clade *Alnus* based germplasm collected from Mirik (A1), Sonada (A5) and loosely Fatak (A2) were there. These places are within the geographic regionof 10-20 km. In the second clade, we have *Alnus* based Sukhiapukhuri (A3) and Ghoom (A4) which are also geographically close. Among the *Casuarina* based population we found them scattered in different clades. *Casuarina* nodules collected from Diamondharbor (C3) went with *Alnus* nodules of Mirik (A1) and Sonada (A5) where as *Casuarina* based *Frankia* of Bakkhali (C4) were clubbing with *Alnus* based *Frankia* of Sukhiapukhuri (A3) and Ghoom (A4). On the other hand *Casuarina* based *Frankia* collected from Jalpaiguri (C1), Digha (C5) and North Bengal

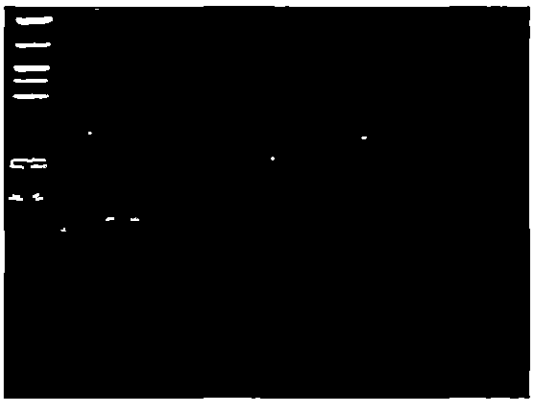


Fig 4.11b. PCR product digested with *Hae*III; Lane 1, 50bp DNA ladder; Lane2-6, A1-A5; Lane 7-11, C1-C5. (for details refer section No. 3.9.2 of MM)

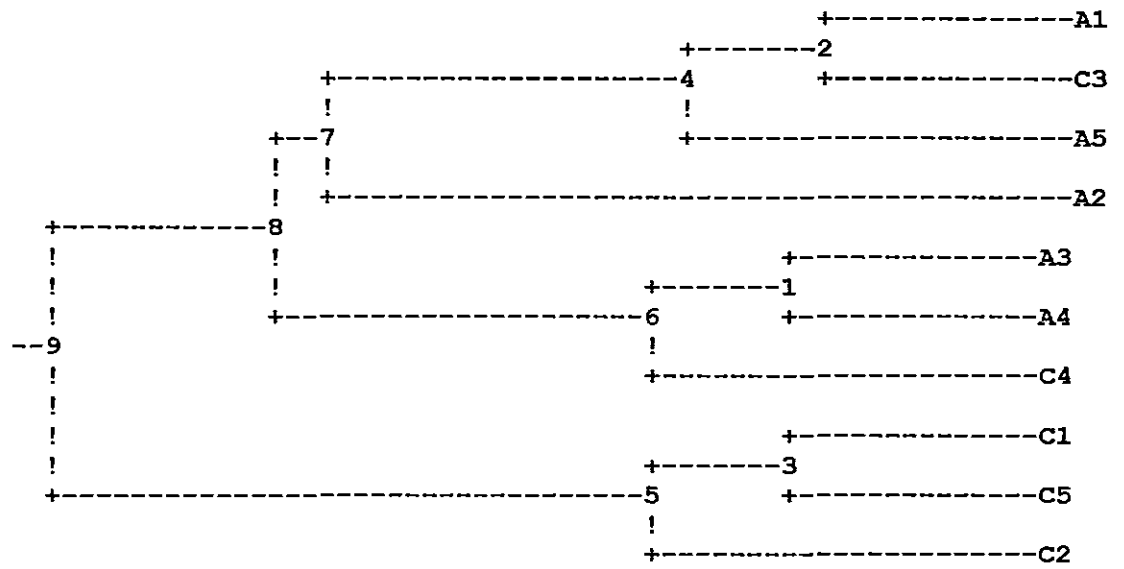


Fig 4.12 Dendrogram Based Nei's (1972) Genetic distance: Method diversity of *Frankia* based on PCR-RFLP results= UPGMA, Modified from NEIGHBOR procedure of PHYLIP Version 3.5

University Campus (C2) comprised of third clade (Fig 4.12).

While from the above result a more or less distinguished pattern emerged in case of *Alnus* based *Frankia*. The *Casuarina* based *Frankia* were places in different clades. The probable reason of *Casuarina* based *Frankia* behaving differently from *Alnus* based *Frankia* could be that all the *Casuarina* plantations in West Bengal are exotic and were perhaps brought from different places.

Chapter 5
CONCLUSION

Before I conclude my work, I must make a summary of it. So, I summarize my whole work in a point wise fashion, which is as followes :

•*Frankia* strains from different parts of West Bengal were isolated from the nodules of *C. equisetifolia* and *A. nepalensis* by a newly standardized isolation technique.

- The culture characteristics and Field Emission Scanning Electron microscopy of the bacterium was performed.
- Effect of different media and hormone concentrations on the germination of seed of *A. nepalensis* were studied.
- The characteristic root hair deformation and visible nodule formation in *A. nepalensis* and *C. equisetifolia* seedlings authenticate the presence of *Frankia* in the medium.
- Physiological characterization of *Frankia* strains were performed.
- Heavy metal resistance profiling of *Frankia* strains were performed and heavy metal induced pigment production of *Frankia* strains was reported.

•Codon usage and codon preferences, G+C composition, effective number of codon (Nc) and codon adaptation index of heavy metal resistance genes of *Frankia* strain EAN1pec, ACN14a and CcI3 were calculated. From these study the life cycle pattern of *Frankia* strains were predicted.

- A new pair of PCR primers specific for distal part of 16s rDNA region of *Frankia* were developed and subsequently, a 520 bp long portion of *Frankia* genome were amplified with those primers followed by PCR-RFLP profiling revealed the diversity of *Frankia* in West Bengal.

At the time of starting of my work, a simple but basic question was in my mind, that was the problem of studying the diversity of *Frankia* and I set my mind to work on it. After starting the work, I found that isolation of *Frankia* was a big problem and there was no

easy technique for isolation of the endophyte from the nodule. Ironically the solution of this question opens up many avenues. I choose a bidirectional approach to find the answer of the diversity related questions. One was strain based work and other was metagenomic work. At this point of study, I decided that I should stick to common parameters only for studying the diversity of both *Alnus* based *Frankia* and *Casuarina* based *Frankia.* I selected standard biochemical and metal resistance parameters for them. On the basis of these results it was found that *Frankia* population of this region was divided in to two major clades. The first clade included the bacterium isolated from *C. equisetifolia* and the second clade included bacterium isolated from *A. nepalensis*. Each clade was again sub divided into two minor clades. This pattern supported the presence of two physiological groups among the bacteria isolated from each host. The results of the metal resistance work were also interesting. *Frankia* strains isolated from *A. nepalensis* were less resistant to the heavy metal salts than the strains isolated from *C. equisetifolia* . It was hypothesized that *Frankia* isolated from *A. nepalensis* facing less heavy metal stress than the *Frankia* isolated from *C. equisetifolia.*

In the year 2007, three *Frankia* whole genome sequences were available in public domain. I took this opportunity to start studying the metal resistance genes of *Frankia* with bioinformatics tools like CodonW, ClustalW, CAI calculator, etc. The metal resistance genes of *Frankia* had high CAI values which was indicating better expression levels. These genes also showed strong codon bias, the result obtained in this study put an additional support to the hypothesis that *Frankia* strain CcI3 is more symbiotic than saprophytic, since they remain largely in the nodules (a protective place for them) and hardly grow in the soil. This kind of work was first of its kind in India.

For metagenomic work, it was found that there was no PCR primer that had the ability to amplify the portion of 16s r-DNA of both *Alnus* based and *Casuarina* based *Frankia.* So, we had developed a new PCR primer for that and found positive results. To study the diversity, the amplicons were digested with restriction enzymes and PCR-RFLP profiles were obtained. Based on those PCR-RFLP profiles, it was found that a

more or less distinguished pattern emerged in case of *Alnus* based *Frankia.* The *Casuarina* based *Frankia* were places in different clades. The probable reason of *Casuarina* based *Frankia* behaving differently from *Alnus* based *Frankia* could be that all the *Casuarina* plantations in West Bengal are exotic and were perhaps brought from different places.

Now, it is the time to answer the question which I had asked at the time of staring of my work. I shall answer it in a positive mood. I have successfully detected the diversity of both *Alnus* based and *Casuarina* based *Frankia* sticking on the common parameters. Although their diversity was totally intrapopulation in origin and do not cross the line of speciation.

REFERENCES

Abeysekera RM, Newcomb W, Silvester WB & Torrey JG (1990) A freeze-fractureelectron microscopic study of *Frankia* in root nodule of *Alnus incana* grown in three oxygen tensions. Can.J.Microbiol 36:97-108.

Akimov V & Dobritsa S (1992) Grouping of *Frankia* strains on the basis of DNA relatedness. Syst Appl Microbiol 15: 372-379.

Akimov V, Dobritsa S & Stupar O (1991) Grouping of *Frankia* strains by DNA-DNA homology: how many genospecies are in the genus *Frankia*? In: Nitrogen Fixation (Polsinelli RM & M Vincenzini eds). Kluwer Academic Publisher, Dordrecht, The Netherlands. pp. 635-636.

Akkermans ADL & Houvers H (1983) Morphology of nitrogen fixers in forest ecosystems. In: Biological nitrogen fixation in forest ecosystems: foundations and applications. (Gordon JC & Wheeler CT eds.), Nijhoff/Junk The Hague.

Akkermans ADL & Hirsch AM (1997) A reconcideration of terminology of *Frankia* research: A need of congruence. Physiol. Plant. 99:574-578.

Alloisio N, Félix S, Maréchal J, Pujic P, Rouy Z, Vallenet D, Medigue C & Normand P (2007) *Frankia alni* proteome under nitrogen-fixing and nitrogen-replete conditions. Physiologia Plantarum. 130 (3): 440–453. (DOI: 10.1111/j.1399-3054.2007.00859.x)

An C, Wills J, Riggsby W & Mullin B (1983) Deoxyribonucleic acid base composition of 12 *Frankia* isolates. Can. J. Bot. 61: 2859-2862.

An C, Riggsby W & Mullin B (1985) Relationships of *Frankia* isolates based on deoxyribonucleic acid homology studies. Int. J. Syst. Bacteriol. 35: 140-146.

An C, Riggsby W & Mullin B (1987) DNA relatedness of *Frankia* isolates ArI4 and EuI1 to other actinomycetes of cell wall type III. The Actinomycetes 20: 50-59.

Banarjee T, Basak S, Gupta SK & Ghosh TC (2004) Evolutionary force inshaping codon and amino acid uses in *Blochmannia floridanus*. J.Biomol. Struct Dyn 22: 13-23.

Bajwa BS (2004) Molecular characterization of *Frankia* and Alder- *Frankia* symbiosis in eastern India. PhD thesis, University of North Bengal.

Baker D, Newcomb W & Torrey JG (1980) Characterization of an ineffective micorhizal microsymbiont, *Frankia* sp. EuII. Can.J.Microbiol. 26:1072-1089.

Baker D (1987) Relationships among pure-cultured strains of *Frankia* based on host specificity. Physiol Plant 70: 245-248.

Baker D & O'Keefe D (1984) A modified sucrose fractionation procedure for isolation of *Frankia* from actinorhizal root nodules and soil samples. Plant Soil 78:23-28.

Bargali K (2011) Actinorhizal plants of Kumaun Himalaya and their ecological significance. Afri. J Plant Sci 5(7):401-406.

Basistha BC, Sharma NP, Lepcha L, Arrawatia ML & Sen A (2010) Ecology of *Hippophae salicifolia* D.Don of temperate and sub alpine forests of North Sikkim Himalayas – a case study. Symbiosis. 50:87-95.

Becana M, Dalton DA, Moran JF, Iturbe-Ormaetxe I, Matamoros MA & Rubio MC (2000) Reactive oxygen species and antioxidants in legume nodules. Physiologia Plantarum 109: 372–381. (doi:10.1034/j.1399-3054.2000.100402.x)

Becking J, de Boer W & Houwink A (1964) Electron microscopy of the endophyte of *Alnus glutinosa.* Ant v Leeuwen. J. Microbiol. Serol. 30: 343-376.

Benoit LF & Berry AM (1990) Methods for production and use of actinorhizal plants in forestry, low-maintenance landscape, and vegetation. In: The biology of *Frankia* and Actinorhizal plants (Schwintzer CR & Tjepkema JD eds) Academic press, San Diego. pp.281-297.

Becking JH (1970) Frankiaceae fam. nov. (Actinomycetales) with one new combination and six new species of the genus *Frankia* Brunchorst 1886, 174. Int. J. Syst. Bacteriol. 20: 201-220.

Beckwith J, Tjepkema JD, Cashon RE, Schwintzer CR & Tisa LS (2002) Hemoglobin in five genetically diverse *Frankia* strains. Can J Microbiol 48:1048–1055. (doi:10.1139/w02-106).

Benson DR & Eveleigh DE (1979) Ultrastructure of the nitrogen fixing symbiont off *Myrica pensylvanica* L. (Bayberry) root nodules. Bot. Gaz 140 (Suppl) S15-S21.

Benson DR & Schultz NA (1990) Physiology and biochemistry of *Frankia* in Culture. In: The biology of *Frankia* and Actinorhizal plants (Schwintzer CR & Tjepkema JD eds). Academic Press, San Diego. pp 107-127.

Benson DR & Silvester WB (1993) Biology of *Frankia* strains, Actinomycete symbionts of Actinorhizal plants. Microbiological Reviews 57: 293-319.

Benson DR, Stephens DW, Clawson ML & Silvester WB (1996) Amplification of 16s rRNA genes from *Frankia* strains in root nodules of *Ceanothus griseus, Coriaria arborea, C. plumose, Discaria toumatou* and *Purshia tridentate.* Appl. Environ. Microbiol. 62: 2904-2909.

Benson DR & Clawson ML (2000) Evolution of the actinorhizal plant symbiosis. In: Prokaryotic nitrogenfixation: A model system for analysis of a biological process (Triplett EW ed.) Wymondham, UK. Horizon Scientific press, pp 207-224.

Berg RH & McDowell L (1987) Endophyte differentiation in *Casuarina* acinorhizae. Protoplasma.136:104-117.

Berg RH & McDowell L (1988) Cytochemistry of the wall of infected cells in *Casuarina* actinorhizae. Can J Bot 66: 2038–2047.

Berry AM & Torrey JG (1983) Root hair deformation in the infection process of

Alnus rubra. Can. J. Microbiol. 61:2863-2876.

Berry AM, Murphy TM, Okubara PA, Jacobsen KR, Swensen SM & Pawlowski K (2004) Novel expression pattern of cytosolic Gln synthetase in nitrogenfixing root nodules of the actinorhizal host, *Datisca glomerata.* Plant Physiology 135: 1849–1862. (doi:10.1104/pp.103.031534).

Berry AM, Mendoza-Herrera A, Guo YY, Hayashi J, Persson T, Barabote R, Demchenko K, Zhang S & Pawlowski K (2011) New perspectives on nodule nitrogen assimilation in actinorhizal symbioses. Functional Plant Biology 38: 645–652. (doi:10.1071/FP11095).

Bikandi J, San Millán R., Rementeria A. & Garaizar J (2004) *In silico* analysis of complete bacterial genomes: PCR, AFLP-PCR, and endonuclease restriction. Bioinformatics 20:798-9. (DOI: 10.1093/bioinformatics/btg491).

Bond G (1983) Taxonomy and distribution of non-legume nitrogen-fixing systems. In: Biological nitrogen fixation in forest ecosystems: foundations and applications. (Gordon JC & Wheeler CT eds.) The Hague: Nijhoff/Junk.

Bosquet J, Girouard E, Strobeck L, Dancik BP & Lalonde M (1989) Restriction fragment length plymorphism in the rDNA region among seven species of *Alnus* and *Betula papyrifera.* Plant and Soil., 118: 231-240.

Bourke RM (1985) Food, coffee and *Casuarina*: an agroforestry system from the Papua New Guinea highlands. Agroforestry Systems 2: 273-279.

Bose D & Sen A (2005) Heavy metal resistance pattern of *Frankia* isolated from sub Himalayan West Bengal. In: Stress Biology: Current Research Scenario (Chakraborty U & Chakraborty BN eds.), Narosa Publication House, New Delhi. pp 154-157.

Bose D & Sen A (2006) Isolation and heavy metal resistance pattern of *Frankia* from *Casuarina equisetifolia* nodules. Indian J Microbiol, 46(1):9-12.

Bose D & Sen A (2007) Heavy metal induced pigment production by *Frankia* isolates from Mirik Hills . Journal of Hill Research , Gangtok. 20(1):36-38.

Bose D, Sur S, Bothra AK & Sen A (2007) Study of the diversity of heavy metal resistance genes and their codon usage profiling. The Icfai Journal of Biotechnology, Icfai University Press, Hyderabad, I(3):49-59.

Bose D, Bajwa B, Bajwa M & Sen A (2011) Physiological characterization of *Frankia* strains isolated from sub-Himalayan West Bengal, India reveals two distinct groups. Biotechnology, 3(1),6-15.

Bosco M, Fernandez MP, Simonet P, Materassi R & Normand P (1992) Evidences that some *Frankia* sp strains are able to cross boundaries between *Alnus* and *Elaeagnus* host specificity groups. Appl. Environ. Microbiol. 58:1569-1576.

Bosco MS, Jamann S, Chapelon C, Simonet P & Normand P (1994) *Frankia* microsymbiont in *Dryas drummondii* nodules is closely related to the microsymbiont of *Coriaria* and genetically distinct from other characterized *Frankia* strains. In: Nitrogen Fixation with Non-legumes (Hegazi MF & Monib M eds.),

The American University in Cairo Press, Cairo. pp. 173-183.

Bottomley WB (1912) The root nodules of *Myrica gale.* Annuls of Botany. Vol XXVI, No. CI. (Downloded from aob.oxfordjournals.org).

Brown AHD & Feldman MW (1981) Population structure of multilocus associations. Proc. Natl. Acad. Sci; 78 (9):5913-5916.

Brewin NJ (2002) Pods and Nods: a new look at symbiotic nitrogen fixing bacteria. Biologist(London, England) 493:113-117.

Buckley G (2000) Fertilizer requirements in 2015 and 2030. IFA, Fertilizer Demand Group, Food and agriculture organization of the United Nations, Rome.

Bumb B & Baanante C (1996) The role of fertilizer in sustaining food security and protecting the environment to 2020. Discussion paper no 17, International Food Policy Research Institute (IFPRI), Washington DC.

Callaham D, Del Tredici P & Torrey JG (1978) Isolation and cultivation in vitro of the actinomycete causing root nodulation in *Comptonia.* Science 199: 899-902.

Callaham D, Newcomb W, Torry JG & Peterson RL (1979) Root hair infection in actinomycetes induced root nodule initiation in *Casuarina, Myrica,* and *Comptonia.* Bot Gaz 140: S51-S59.

Céremonie H, Debelle F, & Fernandez MP (1999) Structural and functional comparison of *Frankia* root hair deforming factor and rhizobiaNod factor. Can J Bot 77: 1293–1301.

Chapin FS, Moilanen L & Kielland K (1993) Preferential use of organic nitrogen for growth by a non-mycorrhizal arctic sedge. Nature 361:150-154.

Chang JO, Kim HB, Kim J, Kim WJ, Lee H, Sun C (2012) Organization of nif gene cluster in *Frankia* sp. EuIK1 strain, a symbiont of *Elaeagnus umbellate.* Arch Microbiol 194:29–34 (DOI 10.1007/ s00203-011-0732-7)

Clawson ML, Bourret A & Benson DR (2004) Assessing the phylogeny of *Frankia*-actinorhizal plant nitrogen-fixing root nodule symbioses with *Frankia* 16S rRNA and glutamine synthetase gene sequences. Mol Phylogenet Evol 31: 131-138.

Coats V, Schwintzer CR & Tjepkema JD (2009) Truncated hemoglobins in *Frankia* CcI3: effects of nitrogen source, oxygen concentration, and nitric oxide. Can J Microbiol 55: 867–873. (doi:10.1139/W09 -042).

Corbin D, Barran L & Ditta G, (1983) Organization and expression of *Rhizobium meliloti* nitrogen fixation genes. Proc. Natl. Acad. Sci. USA 80: 3005-3009.

Dean DR, Bolin JT & Zheng L (1993) Nitrogenase metalioclusters structure, organization and asynthesis. J. Bacteriol. 175: 6737-6744.

Diem, HG & Dommergues YR (1983) The isolation of *Frankia* from nodules of *Casuarina.* Can. J. Bot. 61: 2822-2825.

Diem HG, Gauthier D & Dommergues YR (1983) Isolation of *Frankia* from nodules of *Casuarina equisetifolia.* Can J Microbiol 28: 526-530.

Diem HG & Dommergues YR (1988) Isolation, characterization and cultivation of *Frankia.* In: Biological nitrogen fixation, recent development. (NS Subba Rao eds). Gordon and Breach Sci Publ,

New York. pp227-254.

Diem HG & Dommergues YR (1990) Current and potential uses and management of Casuarinaceae in the tropics and subtropics. In: The biology of *Frankia* and Actinorhizal plants (Schwintzer CR and Tjepkema JD eds), Academic press ,San Diego. pp.317-342.

Dobrista SV & Stupar OS (1989) Genetic heterogenecity among *Frankia* isolates from root nodules of individual actinorhizal plants. FEMS Microbiol. Lett. 58:287-292.

Dommergues YR (1987) The role of biological nitrogen fixation in agroforestry. In: Agroforestry a decade of development (Steppler HA & Nair PKR eds). International Council for Research in Agroforestry Nairobi.

Dos Rei M, Wernisch L & Savva R (2003) Unexpected correlation between gene expression and codon uses bias from microarray data for whole *Escherichia coli* K 12 genome. Nuclic Acid research 31: 6976-6985.

Doyle JJ (1998) Phylogenic perspectives on nodulation: Evolving views of plants and symbiotic bacteria. Trends Plant Sci., 3: 473-478.

Dowson JO (1990) Interaction among actinorhizal and associated plant species. In: The biology of *Frankia* and Actinorhizal plants (Schwintzer CR & Tjepkema JD eds), Academic press, San Diego. pp. 299-316.

Dowson JO, Kowalski DG & Dart PJ (1989) Variation with soil depth, topographic position and host species in the capacity of soils from an Australian locale to nodulate *Casuarina* and *Allocasuarina* seedlings. Plant Soil 118: 1-11.

Earl CD, Ronson CW & Ausubel FM (1987) Genetics and structural analysis of the of the *Rhizobium meliloti fixA*, fixB, fixC and fixX genes. J. Bacteriol. 169:1127-1136.

Elkan GH (1992) Biological Nitrogen Fixation. In Encyclopedia of Microbiology (vol1) Academic Press, pp 285-295.

Fernandez M, Meugnier H, Grimont P & Bardin R (1989) Deoxyribonucleic acid relatedness among members of the genus *Frankia.* Int J Syst Bacteriol 39: 424-429.

Filler WA, Kemp RM, Ng JC, Hawkes TR, Dixon RA & Smithy BE (1986) The *nif*H gene productis required for synthesis or stability of the iron-molobdenum co-factor of nitrogenase from *Klebsiella pneumonium.* Eur.J.Biochem. 160:371-377.

Fischer HM (1994) Genetic regulation of nitrogen fixation in *Rhizobia.* Microbiological review, Sept, pp 352-386.

Fontaine MF, Akancelle S & Torrey JG (1984) Initiation and ontogeny of vesicle of in cultured *Frankia* sp strain HFPAr13. J.Bacteriol. 160: 921-927

Franche C, K Lindstrom & Elmerich C (2009) Nitrogen fixing bacteria associated with leguminous and non-leguminous plants. Plant Soil 321:35-59.

Frank AB (1885) Über die auf Wurzelsymbiose beruhende Ernährung gewisser Bäume durch unterirdische Pilze. Berichte der Deutschen Botanischen Gesellschaft 3: 128-145.

Fuhrmann HJ, Fischer HM & Hennecke H (1985) Mapping of *Rhizobium joponicum nifB-fixBC-*, and *fix A* like genes aqnd identification of the *fixA* promoter. Mol.

Gen. Genet. 199:315-322.

Gauthier D, Diem HG & Dommergues YR (1981) Infectivité et effectivité des souches de *Frankia* isolées de nodules de *Casuarina equisetifolia* et *Hippopha rhamnoides*. C. R. Acad. Sci. Ser. III 293: 489-491.

Gauthier D, Diem HG & Dommergues YR (1984) Tropical and subtropical actinorhizal plants. *Pesq. Agropec. Bras.* 19: 119-36.

Gerber NN & Lechevalier MP (1984) Novel benzo (a) napthacene quinone from an actinomycete: *Frankia* G2 (ORS020604). Can.J.Chem. 62:2818-2821.

Gherbi H, Markmann K, Svistoonoff S, Estevan J, Autran D, Giczey G,Auguy F, Péret B, Laplaze L, Franche C, Parniske M, & Bogusz D (2008) SymRK defines a common genetic basis for plant root endosymbioses with arbuscular mycorrhiza fungi, rhizobia, and *Frankia* bacteria. Proce Nat Acad Sci.105: 4928–4932. (doi:10.1073/pnas.0710618105).

Gouy M & Gautier C (1982) Codon usage in bacteria correlation with gene expressivity. Nucl. Acids. Res, 10 (22), 7055-7074.

Goyal AK, Basistha BC, Sen A & Middha SK (2011) Antioxidant profiling of *Hippophae salicifolia* growing in sacred forests of Sikkim, India. Functional Plant Biology. 38: 697–701.

Grantham R, Gautier C, Gouy M, Jacobzone M & Mercier R (1981) Codon catalog uses is a genome strategy modulated foe gene expressivity. Nucleic Acid Res 9:r43-r47.

Gubler M & Hennecke H (1986) *Fix A,B,*and *C* are essential for symbiotic and freeliving microaerobic nitrogen fixation. FEBS Lett. 200:186-192.

Günther C, Schlereth A, Udvardi M & Ott T (2007) Metabolism of reactive oxygen species is attenuated in leghemoglobin-deficient nodules of *Lotus japonicus.* Molecular Plant-Microbe Interactions 20:1596–1603.doi:10.1094/MPMI-20-12-1596

Hahn D, Lechevalier M, Fischer A & Stackebrandt E (1989) Evidence for a close phylogenetic relationship between members of the genera *Frankia, Geodermatophilus*, and "*Blastococcus*" and emendation of the family Frankiaceae. Syst. Appl. Microbiol. 11: 236-242.

Heckmann AB, Hebelstrup KH, Larsen K, Micaelo NM & Jensen EO (2006) Asingle hemoglobin gene in *Myrica gale* retains both symbiotic and nonsymbiotic specificity. Plant Molecular Biology 61: 769–779. doi:10.1007/s11103-006-0048-1

Hibbs DE & Cromack K Jr. (1990) Actirhizal plants in Pacific Northwest forests. In: The biology of *Frankia* and Actinorhizal plants (Schwintzer CR & Tjepkema JD eds.). Academic press, San Diego. pp 343-363.

Hoagland RD & Arnon DI (1950) The water culture for growing plants without soil.California Agric Exp Stn Circ. pp 347.

Horchani F, Prevot M, Boscari A, Evangelisti E, Meilhoc E, Bruand C, Raymond P, Boncompagni E, Aschi-Smiti S, Puppo A & Brouquisse R (2011) Both plant and bacterial nitrate reductases contribute to nitric oxide production in *Medicago truncatula* nitrogen-fixing nodules. Plant Physiol., 155:1023–1036

Horriere F, Lechevalier MP & Lechevalier HA (1983) *In vitro* morphogenesis and

ultrastructure of a *Frankia* sp. Ar13 (Actinomycetales) from *Alnus rubra* and a morphologically similarisolate (Air12) from *Alnus incana* Subsp. *rugosa.* Can.J.Bot. 61:2843-2854.

Horriere F (1984) *In vitro* physiological approach to classification of *Frankia* isolates of 'The *Alnus* group' based on urease , protease and β-glucosidase activities. Plant Soil. 78: 7-13.

Huss-Danell K & Frej AK (1986) Distribution of *Frankia* in soil from forest and afforestation sites in northern Sweden. Plant Soil 90: 407-418.

Hughes M, Donnelly C, Crozier A & Wheeler CT (1999) Effects of the exposure of roots of *Alnus glutinosa* to light on flavonoid and nodulation. Can. J. Bot; 77: 1-5.

Huguet V, Mergeay M, Cervantes E & Fernandez MP. (2004) Diversity of *Frankia* strains associated to *Myrica gale* in Western Europe: impact of host plant (*Myrica* vs. *Alnus*) and of edaphic factors. Environ Microbiol 6: 1032-1041.

Huguet V, Gouy M, Normand P, Zimpfer JF & Fernandez MP. (2005) Molecular phylogeny of Myricaceae: a reexamination of host-symbiont specificity. Mol Phylogenet Evol 34: 557-568.

Huguet V, Land EO, Casanova JG, Zimpfer JF & Fernandez MP. (2005) Genetic diversity of *Frankia* microsymbionts from the relict species *Myrica faya* (Ait.) and *Myrica rivas-martinezii* (S.) in Canary Islands and Hawaii. Microb Ecol. 49: 617-625.

Huss-Danell K & Bergmann B (1990) Nitrogenase in *Frankia* from root nodules of *Alnus incana* (L.) Moench: immunolocalization of the Fe and MoFe-proteins during vesicle differentiation. New phytol, 116: 443-455.

Ikemura T (1981) Correlation between the abundance of *Escherichia coli* transfer RNa and the occurrence of the respective codons in its protein genes. J.Mol. Biol. 146:1-21.

Iismaa SE, Ealing PM, Scott KF & Watson JM (1989) Molecular linkage of the *nif/fix* and *nod* gene region in *R. leguminosarum bv. trifolii.* Mol.Microbiol. 3:1793-1764.

Jacobsen-Lyon K, Jensen EO, Jorgensen JE, Marcker KA, Peacock WJ & Dennis ES (1995) Symbiotic and non-symbiotic hemoglobin genes of *Casuarina glauca.* The Plant Cell 7: 213–222.

Jamann S, Fernandez M & Moiroud A (1992) Genetic diversity of Elaeagnaceae-infective *Frankia* strains isolated from various soils. Acta Oecolog 13 : 395-405.

Jamann S, Fernandez M & Normand P (1993) Typing method for N2-fixing bacteria based on PCR-RFLP application to the characterization of *Frankia* strains. Mol. Ecol. 2 : 17-26.

Jeong S, Ritchie N & Myrold DD (1999) Molecular phylogenies of plants and *Frankia* support multiple origins of actinorhizal symbioses. Mol. Phylogenet. Evol. 13: 493-503.

Johnson LAS (1982) Notes on Casuarinaceae. J. Adelaide Bot. Gard. 6: 73-87.

Karline S, Marzek J, Campbell A & Kaiser D (2001) Characterization of highly expressed genes of diverse prokaryotic genomes. J.Bacteriol 183:5025-5040.

Knight RD, Freeland SJ & Landweber LF (2001) A simple model based on mutation

and selection explains treands in codon and amino acid uses and GC composition within the across genomes. Genome Biol 2 (4): research0010.1-0010.13;doi10.1186/ gb-2001-2-4-research0010.

Knowlton S, Berry A & Berry JG (1980) Evidence that associated soil bacteria may influence root hair infection of actinorhizal plants by *Frankia*. Can.J.Microbiol.26:971 -977.

Kondas S (1981) *Casuarina equisetifolia.* A multipurpose cash crop in India. In: *Casuarina* ecology, management and utilization. (Midgley SJ, Turnbull JW & Johnston RD eds.),Melbourne: CSIRO.

Kosawang C (2009) PhD Thesis. Umea Plant Science Centre. Sweden.

Ladha JK & Reddy PM (1995) Extension of nitrogen fixation to rice - necessity and possibilities. Geojournal 35: 363–372.

Lafay B, Atherton JC & Sharp PM (2000) Absence of translationally selected synonymous codon uses bias in *Helicobacter pylori.* Microbiology 146: 851-860.

Lancelle SA, Torrey JG, Hepier PK & Callaham DA (1985) Ultrastructure of freeze-substituted *Frankia* strain HFPCCcI3, the actinomycete isolated from root nodules of *Casuarina cunninghamiana.* Protoplasma 127:64-72.

Lalonde M (1978) Confirmation of the infectivity of a freeliving actinimyceteisolated from *Comptonia peregrina*(L.) Coult. Rootnodule. Bot. Gaz. 140(Suppl): S35-S43.

Lalonde M, Knowles R & Devoe IW (1976) Absence of "viod area" in freeze-etched vesicles of the *Alnus crispa* var. *mollis* Fern. Root endophyte. Arch. Microbiol. 107:263-267.

Lalonde M (1979) Immunological and ultrastructural demonstration of nodulation of the European *Alnus glutinosa* (L.) Gaertn. host plant by an actinomycetal isolate from the North American *Comptonia peregrina* (L.) Coult. root nodule. Bot Gaz 140(S), S35-S43.

Lalonde M & Calvert HE (1979) Production of *Frankia* hyphae and spores as an infective inoculant for *Alnus* species. In: symbiotic nitrogen fixation in the management of temperate forests. (Gordon JC, Wheller CT & Parry DA eds) For Res Lab. Oregon State University. Corvallis. pp. 95-110.

Lalonde M, Simon L, Bousquet J & Séguin A (1988) Advances in the taxonomy of *Frankia*: recognition of species *alni* and *elaeagni* and novel subspecies *pommerii* and *vandijkii.* In: Nitrogen Fixation: Hundred Years After (Bothe H & Newton WE eds), Gustav Fischer, Stuttgart. pp. 671-680.

Lechevalier MP, Baker D & Horrire F (1983) Physiology, chemistry, serology and infectivity of two *Frankia* strains isolated from *Alnus incana* subsp *rugosa.* Can.J.Bot. 61:2826-2833.

Lechevalier M (1984) The taxonomy of the genus *Frankia.* Plant Soil 78: 1-6.

Lechevalier MP, Horrire F & Lechevalier HA (1982) Biology of *Frankia* and related organisms. Dev. Ind. Microbiol. 23:51-60.

Lechevalier MP & Lechevalier HA (1984) Taxonomy of *Frankia.* In: Biological, biochemical and biomedical aspects of Actinomycetes. (Ortiz-Ortiz L, Bojalil LF

& Yakoleff V.eds). Academic Press, New York. pp. 575-582.

Lechevalier M & Ruan J (1984) Physiology and chemical diversity of *Frankia* spp. isolated from nodules of *Comptonia peregrina* (L.) Coult. and *Ceanothus americanus* L. Plant Soil 78 : 15-22.

Lechevalier MP & Lechevalier HA (1990) Systamatics, isolation and culture of *Frankia* In: The biology of *Frankia* and Actinorhizal plants (Schwintzer CR & Tjepkema JD eds). Academic Press, San Diego pp 35-60.

Locci R & Sharples GP (1984) Morphology, In: The biology of the actinomycetes. (Goodfellow M, Mordarski M, and Williams TS eds).Academic Press. Inc. New York. pp165-199.

Lumini E, Bosco M & Puppi G (1994) Field performance of *Alnus cordata* Loisel (italian alder) inoculated with *Frankia* and VA-mycorrhizal strains in mine spoil afforestation plots. Soil Biol Biochem 26:659-661.

Maillet F, Poinsot V, André O, Puech-Pagès V, Haouy A, Gueunier M, Cromer L, Giraudet D, Formey D, Niebel A, Martinez EA, Driguez H, Bécard G, & Dénarié J (2011) Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature 469: 58–63. (doi:10.1038/nature09622)

Marechal J, Clement B & Nalin R (2000) A *recA* gene phylogenetic analysis confirms the close proximity of *Frankia* to *Acidothermus*. Int. J. Syst. Evol. Microbiol. 50: 781-785.

Markmann K, Giczey G, & Parniske M (2008) Functional adaptation of a plant receptor-kinase paved the way for the evolution of intracellular root symbioses with bacteria. PLoS Biology 6, e68.

Markowitz VM, Ivanova N, Palaniappan K, Szeto E, Korzeneiwski F, Lykidis A, Anderson I, Mavrommatis K, Kunin V, Garcia Martin H, Dubchak I, Hugenholtz P & Kyrpides NC (2006) An experimental metagenomic data management and analysis system. Bioinformatics 22:e359-e367.

Mastronunzio JE, Tisa LS & Normand P (2008) Comparative secretome analysis suggests low plant cell wall degrading capacity in Frankia symbionts., 47. BMC genomics. http://www.pubmedcentral.nih.gov/arti...

Mastronunzio JE & Benson D (2010) Wild nodules can be broken: proteomics of *Frankia* in field collected root nodules. Symbiosis, 50: 13-26. (DOI 10.1007/s13199-009-0030-1).

McCray Batzli J, Zimpfer JF, Huguet V, Smyth CA, Fernandez M & Dawson JO (2004) Distribution and abundance of infective, soilborne *Frankia* and host symbionts *Shepherdia, Alnus*, and *Myrica* in a sand dune ecosystem. Can. J. Bot. 82: 700-709.

McEwan NR & Gatherer D (1999) Codon indices as a predictor of gene functionality in a *Frankia* operon.Canadian Journal of Botany, 77(9): 1287-1292, (doi10.1139/b99-068).

Miflin BJ & Lea PJ (1977) The pathway of nitrogen assimilation in plants. Progr. Phytochem, 4: 1-26.

Miller IM & Baker DD (1986) Nodulation of actinorhizal plants by *Frankia* strains capable of both root hair infection and intercellular penetration. Protoplasm.

131:82-91.

Misra AK & Verghses SK (2004) Some aspects of actinorhizal symbiosis. Ind. J. Microbiol. 44(4):235-246.

Misra AK & Sen A (2011) *Frankia* and actinorhizal symbiosis. In: Mirroorganism in sustainable agriculture and biotechnology. .(Satyanarayana T *et* al eds). (DOI 10.1007/978-94-007-2214-9-7).Springer. (In press).

Mirza M, Akkermans W & Akkermans A (1994) PCR-amplified 16S rRNA sequence analysis to confirm nodulation of *Datisca cannabina* L. by the endophyte of *Coriaria nepalensis* Wall. Plant and Soil 160: 147-152.

Mort A, Normand P & Lalonde M (1983) 2-o-methyl-D-mannose, a key sugar in the taxonomy in the taxonomy of *Frankia.* Can. J. Microbiol, 29:993-1002.

Mullis K, Faloona F, Scharf S, Saiki R, Horn G & Erlich H (1986) Specific enzymatic amplification of DNA *in vitro*: The polymerase chain reaction. Cold Spring Harbor Symp. Quant. Biol. 51:263-273.

Murry MA, Konopka AS, Pratt SD & Vandergon TL (1997) The use of PCR-based typing methods to assess the diversity of *Frankia* nodule endophytes of the actinorhizal shrub *Ceanothus*. Physiol. Plant. 99: 714-721.

Myrold DD (1994) *Frankia* and the Actinorhizal symbiosis. In: Methods of soil Analysis, Part 2. Microbiological and Biochemical Properties-SSSA Book Series, no.5. Soil science society of America. USA.pp 291-338.

Nazaret S, Cournoyer B, Normand P & Simonet P(1991) Phylogenetic relationship s among *Frankia* genomic species determined by the use of amplified 16s rDNA sequence. J.Bacteriol. 173:4072-4078.

Navarro E, Jaffre T, Gauthier D, Gourbiere F, Rinaudo G, Simonet P & Normand P (1999) Distribution of *Gymnostoma* spp. microsymbiotic *Frankia* strains in New Caledonia is related to soil type and to host-plant species. Mol. Ecol. 8: 1781-1788.

Nei M (1972) Genetic distance between populations. American Naturalist 106: 283-292.

Nei M (1987) Molecular evolutionary genetics New York; Columbia University Press.

Newcomb W, Callaham D, Torry JG & Peterson RL (1979) Morphogenesis and fine structure of the actinomycetous endophyte of nitrogen fixing root nodule of *Comptonia peregrina.* Bot.Gaz. 140 (Suppl) S22-S34.

Newcomb W & Wood SM (1987) Morphogenesis and fine structure of *Frankia (Actinomycetales):* the microsymbiont of nitrogen fixing actinorhizal root nodules. Int. Rev. Cytol. 109:1-88.

Nick G, Paget E, Simonet P, Moiroud A & Normand P (1992) The nodular endophytes of *Coriaria* spp. form a distinct lineage within the genus *Frankia.* J. Mol. Ecol. 1 (3), 175-181.

Niemann J & Tisa LS (2008) Nitric Oxide and Oxygen Regulate Truncated Hemoglobin Gene Expression in *Frankia* Strain. CcI3. J. Bacteriol. 190: 7864-7867; doi:10.1128/JB.01100-08

Nies DH (1999) Microbial heavy-metal resistance. Appl Microbiol Biotechnol 51: 730-750

Normand P & Lalonde M (1982) Evaluation of *Frankia* strains isolated from provenances of two *Alnus* species. Can. J. Microbiol. 28: 1133-1142.

Normand P & Bousquet J (1989) Phylogeny of *nifH* sequence in *Frankia* and other nitrogen-fixing microorganisms. J.Mol. Evol., 29:436-447

Normand P, Orso S, Cournoyer B, Jeannin P, Chapelon C, Dawson J, Evtushenko L& Mishra AK (1996). Molecular phylogeny of the genus *Frankia* and related genera and emendation of thepropagation of the sea buckthorn (*Hippophae rhamnoides*) *Erdo* 31(7):309-312.

Normand P, Cournoyer B, Nazaret S & Simonet P (1994) Analysis of a ribosomal RNA operon in actinomycete *Frankia*. Gene. 111: 119-124.

Normand P, Orso S & Cournoyer B (1996) Molecular phylogeny of the genus *Frankia* and related genera and emendation of the family Frankiaceae. Int. J. Syst. Bacteriol. 46: 1-9.

Normand P & Fernandez MP (2006) Evolution and Diversity of *Frankia* In: Prokaryotic endosymbionts in plants. (K Pawlowski ed). Springer.

Normand P, Lapierre P, Tisa LS, Gogarten JP, Alloisio N, Bagnarol E, Bassi CA, Berry AM, Bickhart DM, Choisne N, Couloux A, Cournoyer B, Cruveillar S, Daubin V, Demange N, Francino MP, Goltman E, Huang Y, Kopp OR, Labarre L, Lapidus A, Lavire C, Marachal J, Martinez M, Mastronunzio JE, Mullin BC, Niemann J, Tania PP, Zoe Ruby R, Schenowitz C, Sellstedt A, Tavares F, Tomkins JP, Vallenet D,Valverde C, Wall LG, Wang Y, Medigue SC & Benson D (2007) Genome characteristics of facultatively symbiotic *Frankia* sp. strains reflect host range and host plant biogeography. Genome Res. January; 17(1): 7–15.

Obertello M, Wall L, Laplaze L, Nicole M, Auguy F, Gherbi H, Bogusz D & Franche C (2007) Functional analysis of the metallothionein gene *cgMT1*isolated from the actinorhizal tree *Casuarina glauca.* Molecular Plant-Microbe Interactions 20: 1231–1240. doi:10.1094/ MPMI-20-10-1231

Ochman H & Wilson AC (1987) Evolution in bacteria: evidence for a universal substitution rate in cellular genomes. J Mol Evol 26: 74-86.

Oldroyd GE, Harrison MJ, & Paszkowski U (2009) Reprogramming plant cells for endosymbiosis. Science 324: 753–754. (doi:10.1126/science.1171644).

Opden Camp R, Streng A, De Mita S, Cao Q, Polone E, Liu W, Ammiraju JS, Kudrna D, Wing R, Untergasser A, Bisseling T & Geurts R (2011) LysMtype mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume *Parasponia.* Science 331:909–912. (doi:10.1126/science.1198181).

Parsons R, Silvester WB, Harris S, Gruijters WTM & Bullivant S (1987) *Frankia* vesicles provides inducible and absolute oxygen protection for nitrogenase. Plant. Physiol. 83:728-731.

Patyk A & Reinhard G (1997) Energy and material flow analysis of fertiliser production and supply, In: 4th Symposium for Case Studies in Life Cycle Analysis, Brussels, December 1996, pp. 73–86.

Pawlowski K & Bisseling T (1996) Rhizobial and actinorhizal symbioses: what are the shared features? The Plant Cell 8:1899–1913.

Pawlowski K, Jacobsen KR, Alloisio N, Denison RF, Klein M, Winzer T, Sirrenberg A, Guan C & Berry AM (2007) Truncated hemoglobins in actinorhizal nodules of *Datisca glomerata.* Plant Biology 9: 776–785. doi:10.1055/s-2007-965258

Pawlowski K, Didier AE, Bogusz B, Ribeiro AC & Berry AM (2011) Progress on research on actinorhizal plants Functional Plant Biology, 38: 633–638.

Peden J (1999) Analysis of codon usage. Ph.d Thesis, University of Nottingham, U.K

Pesole G, Gissi C, Lanave C & Saccone C (1995) Glutamine synthetase gene evolution in bacteria. Mol Biol Evol. 12 : 189-197.

Peters JW & Szilagyi RK (2006) Exploring new frontiers of nitrogenase structure and mechanism. Curr. Opin.Chem. Biol. 10:101-108

Pommer EH (1956) Beiträge zur Anatomie und Biologie der Wurzelknöllchen von *Alnus glutinosa* Gaertn. Flora 14: 603-634.

Pommer EH (1959) Uber die Isolierung des Endophyten aus den Wurzelknöllchen *Alnus glutinosa* Gaertn. und uber erfolgreiche re-Infektions Versuche. Ber. Dtsch. Bot. Ges. 72:138-150.

Quispel A (1988) Hellriegel and Wilfarth's discovary of (symbiotic)nitrogen fixation hundred years aoo. In: Nitrogen Fixation: Hundred years after.(Bothe H, deBruijn FJ & Newton WE eds). Fischer, Stuttgart. pp3-12.

Quispel A (1990) Discoveries, discussion and trends in research on actinorhizal root nodule symbioses before 1978. In: The biology of *Frankia* and Actinorhizal plants (Schwintzer CR & Tjepkema JD eds) Academic press, San Diego. pp.15-33.

Raman N & Elumalai S (1991) Studies on mycorrhizal and Actinorhizal association in *Casuarina equisetifolia* in Coramandal costal region. .Tropical Forestry. Vol7(II).

Richards JW, Krumholz GD, Matthew SC & Tisa LS (2002) Heavy metal resistance patterns of *Frankia* strains. Appl Environ Microbiol 68: 923-927.

Ridgway KP, Marland LA & Harrison AF (2004) Molecular diversity of *Frankia* in root nodules of *Alnus incana* grown with inoculum from polluted urban soils. FEMS Microbiol. Ecol. 50: 255-263.

Rouvier C, Nazaret S, Fernandez MP, Picard B, Simonet P & Normand P (1992) *rrn* and *nif* intergenic spacers and isozyme patterns as tools to characterize *Casuarina* infective *Frankia* strains. Acta Oecol. 13: 487-495.

Rouvier C, Prin Y, Reddell P, Normand P & Simonet P (1996) Diversity among *Frankia* strainsnodulating members of the family Cacuarinaceae in Australia revealed by PCR and restriction fragmeny length polymorphism analysis with crushed root nodules. Appl.Evviron Microbiol. 62:979-985.

Rosswall T (1981) The Biogeochemical Nitrogen Cycle. In: Some perspectives of the major biogeochemical cycles. (GE Likens eds), Royal Swedish Academy of Science.

Rosendahl L & Huss.Danell K (1988) Effects of elevated oxygen tention on acetylene reduction in *Alnus incana Frankia*

symbioses. Physiol. Plant. 74:89-94.

Rozan S & Skaletsky HJ (2000) Primer 3 on the WWW for the general use and for the biologist programmers. In: Bioinformatics methods and protocols: Methods in Molecular Biology, (Krawetz S and Misener S eds),Human Press, Totowa, NJ, pp 365-386.

Rubio LM & Ludden PW (2008) Biosynthesis of the iron molybdenum cofecter of nitrogenase. Ann. Rev. Microbiol. 62:93-111.

Ruvkun GB, Sundaresan V & Ausubel F (1982) Directed transposon Tn5 mutagenesis and complemantation analysis of *Rhizobium meliloti* symbiotic nitrogen fixation genes. Cell 29:551-559.

Saitou N & Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Sambrook J & Russell DW (2001) Molecular Cloning . A laboratory manual (3rd eds) vol 3, Appendix 1. A1.19.

Sarma G, Sen A, Varghese R & Misra AK (1998) A novel technique for isolation of *Frankia* and generation of single spore culture. Can J Microbiol 44: 490-492.

Sasakura F, Uchiumi T, Shimoda Y, Suzuki A, Takenouchi K, Higashi S & Abe M (2006) A class 1 hemoglobin gene from *Alnus firma* functions in symbiotic and nonsymbiotic tissues to detoxify nitric oxide. Molecular Plant-Microbe Interactions 19: 441–450. (doi:10.1094/ MPMI-19-0441).

Schwintzer CR (1990) Spore positive and spore negative nodules. In: The biology of *Frankia* and Actinorhizal plants (Schwintzer CR & Tjepkema JD eds). Academic Press, San Diego pp 177-193.

Scholz V (1997) Methods for calculating the energy demand of vegetable products presented on solid biofuels, *Agrartechnische Forschung* 3 : 11–18.

Schubert M, Melnikova AN, Mesecke N, Zubkova EK, Fortte R, Batashev DR, Barth I, Sauer N, Gamalei YV, Mamushina NS, Tietze L, Voitsekhovskaja OV & Pawlowski K (2010) Two novel disaccharides, rutinose and methylrutinose, are involved in carbon metabolism in *Datisca glomerata.* Planta 231: 507–521. doi:10.1007/s00425-009-1049-5.

Schubert M, Koteeva NK, Wabnitz PW, Santos P, Büttner M, Sauer N, Demchenko & Pawlowski K (2011) Carbon partitioning in roots and nitrogen-fixing root nodules of *Datisca glomerata.* Planta 233, 139–152. (doi:10.1007/s00425 -010-1285-8).

Schultz NA & Benson DR (1989) Developmental potential of *Frankia* vesicles. J. Bacteriol. 171:6873-6877.

Sen A (1996) Electron microscopy and molecular biology of *Frankia.* PhD Thesis. North Eastern Hill University, Shillong, India

Sen A, Sur S, Bothra AK, Benson DR, Normand P and Tisa LS (2008) The implication of life style on codon uses pattern and predicted highly expressed genes for three *Frankia* genomes. Antonie van Leeuwenhoek (DOI 10. 1007//s10482-007-9211-1).

Sen A, Sur S , Tisa LS, Bothra AK,Thakur S & Mondal UK (2010) Homology modelling of the *Frankia* nitrogenase iron

protein strains. Symbiosis. 2010, Volume 50,(1-2), 37-44.

Sen A, Thakur S, Bothra AK, Sur S & Tisa LS (2011) Identification of TTA codon containing genes in *Frankia* and exploration of the role of tRNA in regulating these genes. Arch. of mircobiol. DOI 10.1007/s002030-011-0731-8.

Shannon CE (1948) A mathematical theory of communication. Bell System technical Journal 27: 379-423 and 623-656.

Sharma E & Ambasht RS (1986) Root nodule age class transition, production and decomposiotion in an age sequence of *Alnus nepalensis* plantation stands in the Eastern Himalayas ; Journal of Applied Ecology. 23: 689-701.

Sharp PM & Li WH (1987) The codon adaptation index – a measure of directional synonymous codon usage bias and its potential applications. Nucleic Acid Res 15: 1281-1295.

Silvester WB, Silvester JK & Torry JG (1990) Adaptation of nitrogenase to varying oxygen tension and the role of the vesicle in root nodules of Alnus incana ssp rugosa. Can.J. Bot. 66:1772-1779.

Silvester WB, Harris SL & Tjepkema JD (1990) Oxygen regulation and haemoglobin. In: The biology of *Frankia* and Actinorhizal plants (Schwintzer CR & Tjepkema JD eds). Academic Press, San Diego pp 157-176.

Silver S & Phung LT (1996) Bacterial Heavy metal resistance: new surprises. Ann. Rev. of Microbiol, Vol. 50, pp 753-789.

Silver WS (1964) Root nodule symbiosisin endophyte of *Myrica cerifera*. J. Bacteriol. 87:416-421.

Simonet P, Normand P, Moiroud A & Lalonde M (1985) Restriction enzyme digestion patterns of *Frankia* plasmid. Plant soil. 188:13-22.

Simonet P, Grosjean MC, Misra AK, Nazaret S, Cournoyer B & Normand P (1991) *Frankia* genus specific characterization by polymerase chain reaction. Appl. Environ. Microbiol. 57:3278-3286.

Simonet P, Bosco M, Chapelon C, Moiroud A & Normand P (1994) Molecular characterization of *Frankia* microsymbionts from spore positive and spore negetive nodules in a natural alder strand. Appl Environ. Microbiol 57:3278-3286

Smolander A (1990) *Frankia* populations in soils under different tree species with special emphasis on soils under *Betula pendula*. Plant Soil 121:1-10.

Smolander A & Sundman V (1987) *Frankia* in acid soils of forests devoid of actinorhizal plants. Physiol. Plant. 70: 297-303.

Soltis D, Soltis P & Morgan D (1995) Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. Proc. Nat. Acad. Sci. USA 92: 2647-2651.

Spain A & Alm E (2003) Implications of Microbial Heavy Metal Tolerance in the Environment. Reviews in Undergraduate Research, 2:1-6.

Sur S, Sen A & Bothra A (2006) Codon usage profile and analysis of intergenic association of *Frankia* EuIK1 *nif* genes. Ind. J. Microbiol. 46(3): 281-287.

Sur S, Sen A & Bothra A (2007) Mutational drift prevails over translational efficiency in *Frankia nif* operon. Ind. J. Biotech. 6:321-328.

Sur S, Bothra AK & Sen A (2010) Symbiotic nitrogen fixation- A bioinformatics perspective. Biotechnology. 9(3): 257-273.

Swensen SM (1996) The evolution of actinorhizal symbioses: evidence for multiple origins of the symbiotic association. Amer J. Bot 83:1503–1512. doi:10.2307/2446104

Tavares F, Santos CL & Sellstedt A (2007) Reactive oxygen species in legume and actinorhizal nitrogen-fixing symbioses: the microsymbiont's response to an unfriendly reception. Physiologia Plantarum 130: 344 –356. (doi:10.1111/j.1399-3054.2007.00933.x)

Thiagalingam K (1983) Role of *Casuarina* in agroforestry. In: *Casuarina* ecology, management and utilization. (Midgley SJ, Turnbull JW & Johnson RD. eds.) Melbourne: CSIRQ.

Tisa LS (1998) Calcium transport by *Frankia* sp. Curr Microbiol, 37:12-16.

Tisa LS & Ensign JC (1987) Isolation and nitrogenase activity of vesicle from *Frankia* sp strain EAN1pc, Can.J.Bot 77:1257-1260.

Tisa LS & Ensign JC (1988) Evidance for adenylate nucleotide trnsport (ATP-ADP translocation) in vesicles of *Frankia* sp strain EAN1pc. J.Bacteriol. 170:3053-3057.

Tisa LS, Chaval MS,Krumholz GD & Richards JW (1999) Antibiotic resistance pattern of *Frankia* strains. Can. J. Bot. 77: 1257-1260.

Tjepkema JD & Torry JG (1979) Symbiotic nitrogen fixation in actinomycete-nodulated plants. Preface Bot Gaz Suppl.140: i-ii.

Tjepkema JD, Cashon RE, Beckwith J & Schwintzer CR (2002) Hemoglobin in *Frankia*, a nitrogen-fixing actinomycete. Appl Environ Microbiol 68: 2629–2631. (doi:10.1128/AEM.68.5.2629-2631.2002).

Tortosa RD & Cusato M (1991) Effective nodulation of rhamnaceous actinorhizal plants induced byair dry soils, Plant soil, 131:229-233.

Torrey JG & Callaham D (1982) Structural features of the vesicles of *Frankia* sp CpII in culture. Can.J.Microbiol. 28:749-757.

Valverde C & Wall LG (1999) Regulation of nodulation in *Discaria trinervis* (Rhamnaceae) - *Frankia* symbiosis. New Phytol; 141: 345-354.

Van Dijk C & Merkus E (1976) A microscopic study of the development of a spore-like stage in the life cycle of the root nodule endophyte of *Alnus glutinosa* (L.) Vill. NewPhytol. 77:73-90.

Ventura M, Canchaya C, Tauch A, Chandra G, Gerald FF, Keith FC & van Sinderen D (2007) Genomics of *Actinobacteria*: Tracing the Evolutionary History of an Ancient Phylum Microbiol Mol Biol Rev. 71(3): 495–548. (doi:10.1128/MMBR.00005-07).

Verghese S & Misra AK (2002) *Frankia*–actinorhizal symbiosis with special reference to host – microsymbiont relationship. Current Science, 83 (4,25) :402-408.

von Tubeuf K (1895) Pflanzenkrankheiten durch Kryptogame Parasiten verursacht. Verlag J Springer, Berlin.

Waksman SA (1950) The Actinomycetes, their nature, occurrence, activities, and importance. Percaline Rouge, Waltham, Mass., USA.

Wall LG (2000) The actinorhizal symbiosis. J. Plant growth Regul, 19: 167-182.

Wayne LG, Brenner DJ & Colwell RR (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37: 463 -464.

Weber A, Smolander A, Nurmiaho-Lassila E & Sundman V (1988) Isolation and characterization of *Frankia* strains from *Alnus incana* and *Alnus glutinosa* in Finland. Symbiosis. 6:97-116.

Weast RC (1984) CRC handbook of chemistry and physics, 64 edn. CRC, Boca Raton, Fla.

Weaver W & Shannon CE (1949) The mathematical theory of communication. Urbana Illinois; University of Illinois.

Wheeler CT & Miller IM (1990) Current and potential uses of actinorhizal plants in Europe. In: The biology of *Frankia* and Actinorhizal plants (Schwintzer CR & Tjepkema JD eds). Academic press, San Diego. pp 365-389.

Wright F (1990) The effective number of codons used in a gene, Gene, 87,(1), 23-29.

Woese CR (1987). Bacterial evolution. Microbiol Rev 51: 221-271.

Woronin MS (1866) Uber die bei der Schwarzerle (*Alnus glutinosa*) und bei der gewöhnlichen Garten-Lupine (*Lupinus mutabilis*) auftretenden Wurzelanschwellungen. Mémoires de l'Academie Impériale des Sciences de St. Petersbourg, VII Ser. 10: 1-13.

Wu G, Culley DE & Zhang W (2005) Predicted highly expressed genes in the genomes of *Streptomyces coelicolor* and *Streptomyces avermitilis* and the implications of their metabolism. Microbiology 151:2175-2187.

ACADEMIC ACHIEVEMENTS

Paper published from the thesis

1. **Debadin Bose** and Arnab Sen (2005) 'Heavy metal resistance pattern of *Frankia* isolated from sub Himalayan West Bengal'. In: Stress Biology: Current Research Scenario (Chakraborty U and Chakraborty BN eds), Narosa Publication House, New Delhi, pp 154-157.

2. **Debadin Bose** and Arnab Sen (2006). 'Isolation and heavy metal resistance pattern of *Frankia* from *Casuarina equisitifolia* nodules'. Indian Journal of Microbiology, New Delhi. vol 46(1),pp- 9-12

3. **Debadin Bose** , Saubashya Sur, Asim Kr. Bothra and Arnab Sen (2007) 'Study of the diversity of heavy metal resistance genes and their codon usage profiling' The Icfai Journal of Biotechnology, Icfai University Press, Hyderabad, Vol I, No 3, pp 49-59.

4. **Debadin Bose** and Arnab Sen (2007). 'Heavy metal induced pigment production by *Frankia* isolates from Mirik Hills '. Journal of Hill Research , Gangtok. vol 20, no-1, pp 36-38.

5. **Debadin Bose**, B. Bajwa, M. Bajwa and A Sen (2011). "Physiological characterization of *Frankia* strains isolated from sub-Himalayan West Bengal, India reveals two distinct groups". Biotechnology, Vol 3, No 1, pp 6-15.

Technology Generated

1. A nobel technique for isolation of *Frankia* in pure culture.

Paper accepted for presentation in International Seminar in overseas for presentation

1. **Debadin Bose**, B. Bajwa, M. Bajwa and A Sen. "Physiological characterization of *Frankia* strains isolated from sub-Himalayan West Bengal, India reveals two distinct groups" In 16th International Meeting on *Frankia* and Actinorhizal plants & International Symposium on Franineae in city of Porto, Portugal, on 5th to 8th September, 2010.

Paper presented in International Seminar in overseas:

1. **Debadin Bose**, S Sur, AK Bothra and A Sen (2010), "Evolution of genes for heavy metal resistance in *Frankia* -A bioinformatics approach", in Annual Botanical Conference 2010, Rajshahi, Bangladesh; on 11th December, 2010, organized by the Department of Botany, University of Rajshahi, Bangladesh.

Appendix –I

Composition of Buffer

Composition of the Buffers used

1. DNA extraction buffer (C-TAB Extraction buffer)

Ingredients

2% (W/V) C-TAB (Hi-media Cat#RM164)

1.4M NaCl (Hi-media Cat#1150)

20mM EDTA (pH-8.0) (Hi-media Cat#RM1197)

100mM Trizma Base (pH-8.0) (Sigma Cat#T1503)

1% Polyvinylpyrrolidone (PVP)(W/V) (Sigma Cat#P5288)

0.2% β-Mercaptoethanol (Hi-media Cat#RM2895), added just before use.

Procedure

12.11 grams of molecular biology grade Trizma Base (Sigma Cat#T1503, Tris (hydroxymethyl) aminomethen, $C_4H_{11}NO_3$, MW- 121.1) was dissolved in 600 ml of sterile double distilled water and the pH is adjusted to 8.0. The solution was made upto 800 ml with sterile double distilled water and divided into two parts; to one part 7.44 gm of EDTA (Ethylenediaminetetra acetic acid, $C_{10}H_{14}N_2Na_2O_8.H_2O$) was added. In other part 81.82gm of Sodium chloride (NaCl), 20gm of C-TAB (Hexadecyl trimethyl ammonium bromide,$C_{19}H_{42}NBr$) was added. Then both the solutions were mixed and to it 1% Polyvinylpyrrolidone was added. The volume was made upto 1lit with sterilized double distilled water. β -Mercaptoethanol (0.2%) was added just before the use of the buffer.

2. DNA Loading buffer Type III(6X concentration) (Fermentas Cat#R0611)

Ingredients

0.25% Bromophenol Blue (Hi-media Cat#RM914)

0.25% Xylene cyanol FF (Hi-media Cat#RM1877)

30% Glycerol (Hi-media Cat#MB060) in Double distilled water.

Procedure

Two and half gram of Bromophenol Blue and Xylene cyanol was dissolved in 1000 ml of 30% Glycerol in Double distilled water.

3. TE- buffer (Tris EDTA Buffer, pH-8)

Ingredients

10mM Tris Trizma Base (pH-8.0) (Sigma Cat#T1503).

10mM EDTA (pH-8) (Hi-media Cat#RM1197).

Sterile double distilled water.

Procedure

1.21 gm of molecular biology grade Trizma Base (Sigma Cat#T1503, Tris (hydroxymethyl) aminomethen, $C_4H_{11}NO_3$, MW- 121.1) was dissolved in 400 ml of sterile double distilled water and pH was adjusted with concentrated Hydrochloric acid (Hi-media, Cat#RM5955) to 8.0 and sterilized by autoclaving. Similarly 0.372 gm of Di-sodium salt of EDTA (Ethylenediaminetetra acetic acid, $C_{10}H_{14}N_2Na_2O_8.H_2O$) was dissolved in 400 ml of double distilled water. The solution was stirred properly and the pH was adjusted with sodium hydroxide pellets (NaOH, Hi-media, Cat#RM1183) sterilized by autoclaving. Both the solutions were then mixed and the volume was made upto 1lit sterilized double distilled water.

4. TBE- buffer (Tris Borate EDTA Buffer, pH-8) (5X Concentration)

Ingredients

Tris (Trizma Base, Sigma Cat#T1503) 54 gm/lit.

Boric acid (Hi-media Cat#MB007) 27.5 gm/lit.

20 mL of 0.5M EDTA (Hi-media Cat#RM1197).

Sterile double distilled water.

Procedure

54 gm of molecular biology grade Trizma Base (Sigma Cat#T1503, Tris (hydroxymethyl) aminomethen, $C_4H_{11}NO_3$, MW- 121.1) and 27.5 gm of Boric acid (Hi-media Cat#MB007) was dissolved in 800mL of sterile double distilled water. 20 mL of 0.5 M EDTA (pH 8.0) was added. The volume was adjusted up to 1lit with sterile double distilled water.

The working concentration of TBE buffer is 1X, so the 5X stock solution is diluated with sterile distilled water to 1X.

5. Taq buffer (10X Concentration, supplied with Taq polymerase, Finnzymes Cat#F501L)

Ingredients

10 mm Tris-HCl (pH – 8.8).

1.5mM $MgCl_2$(Hi-media Cat# MB040).

50 mM KCl. (Hi-media Cat# RM697).

0.1% Triton X-100. (Hi-media Cat# MB031).

6. Washing solution:

Ingredients

70% ethanol (E Merck Cat# 101076HBD).

10mM Ammonium acetate (Hi-media, Cat#MB033)

7. PBS- PhosphateBuffered saline (pH-7.4)

137 mM NaCl (Hi-media Cat#1150).

2.7mM KCl (Hi-media Cat# RM697).

10mM Na_2HPO_4(Hi-media Cat#RM 1416).

1.8mM KH_2PO_4(Hi-media Cat#RM 2951).

Procedure

8 gm of NaCl, 0.2g of KCl, 1.44gm of Na_2HPO_4 and 0.24gm of KH_2PO_4 were dissolved in 800mL of sterile double distilled water. The pH was adjusted to 7.4 with HCl. Volume was made up to 1 lit with sterile double distilled water.

8. Peeling Buffer (pH-7.4):

NaCl (Hi-media Cat#1150) – 8gm.

KCl (Hi-media Cat# RM697) – 0.2gm.

Na_2HPO_4(Hi-media Cat#RM 1416) – 1.44gm.

KH_2PO_4(Hi-media Cat#RM 2951) – 0.24gm.

Polyvinyl pyrollidone (PVPP) (Sigma Cat#P5288) - 30 gm.

Procedure

The compounds, except PVPP were dissolved in 800mL of sterile double distilled water and the pH was adjusted to 7.4. 30 gm of Polyvinyl pyrollidone (PVPP) was added to this solution. The final volume was made up to 1lit with sterile double distilled water and autoclaved.

Appendix –II

Composition of Medium

	Qmod (Lalonde and Calvert, 1979)	DPM (Baker and O'keefe, 1984)
Macronutrients (gL^{-1})		
K_2HPO_4	0.3	-
KH2PO4	-	1.0
$NaH_2PO_4, 2H_2O$	0.23	-
$MgSO_4,7H_2O$	0.2	0.1
KCl	0.2	-
$CaCl_2$	-	0.01
Yeast extract	0.5	-
Peptone	5.05	-
Lecithin	0.04	-
Sodium propionate	-	1.2
Glucose	10.0	-
Micronutrients and iron (mgL^{-1})		
H_3BO_3	1.5	2.86
$MnSO_4,H_2O$	0.8	-
$MnCl_2$	-	1.8
$ZnSO_4,7H_2O$	0.6	0.22
$CuSO_4,5H_2O$	0.1	0.08
Na_2MoO_4	-	0.025
$(NH_4)_8(Mo_7O_2)_4,4H_2O$	0.2	-
$CoSO_4,7H_2O$	0.025	-
$CoCl_2.7H_2O$	-	0.025
$C_6H_8O_7,H_2O$	10	-
$C_8H_8O_7Fe,3H_2O$	10	-
Fe Na EDTA	-	13.20

F-medium (Simonet *et al.*, 1985) pH = 7.00.

Constituents	Stock solution. (gm/lit)	Working solution (µM/lit)
Macronutrients	**10x**	
KH_2PO_4	5.0	2871
$MgSO_4$	2.0	1148
$CaCl_2,2H_2O$	1.0	2127
D-glucose	100.0	5550
Casein hydrolysate	40.0	
Yeast extract	0.50	
Oligo quispel	**1000X**	
H_3BO_3	1.50	24.3
$MnSO_4, H_2O$	0.80	4.70
$ZnSO_4,7H_2O$	0.60	2.10
$CuSO_4,5H_2O$	0.10	0.40
$(NH_4)_8Mo_7O_2,4H_2O$	0.20	0.16
$CoSO_4,7H_2O$	0.025	0.09
Iron	**1000X**	
$C_8H_8O_7,H_2O$	10.00	47.6
$C_8H_8O_7Fe,3H_2O$	10.00	33.5
Vitamines	**1000X**	
Thiamine HCl	0.01	0.03
Nicotinic acid	0.05	0.04
Pyridoxine HCl	0.05	0.24
Tween 80		1ml

OS-1 medium (Dobritsa and Stupar, 1989)

Constituents	Stock solution. (gm/lit)	Working solution (µM/lit)
Macronutrients	**10X**	
KH_2PO_4	1.36	999.00
$MgSO_4$	3.38	3039.00
Micronutrients	**1000X**	
H_3BO_3	2.86	46.30
$MnCl_2,4H_2O$	1.81	9.10
$ZnSO_4,7H_2O$	0.22	0.80
$CuSO_4,5H_2O$	0.08	0.30
Na_2MoO_4	0.025	0.01
$CoCl_2,7H_2O$	0.025	0.01
Iron		
$FeSO_4,7H_2O$	10.008	370.00
Na_2EDTA	13.41	36.00

Hoagland solution (pH=7, Nitrogen free) (Hoagland and Arnon, 1950)

Constituents	Stock solution. (gm/lit)	Working solution (µM/lit)
Macronutrients	**10X**	
KH_2PO_4	1.36	999.00
$MgSO_4$	3.38	3039.00
Micronutrients	**1000X**	
H_3BO_3	2.86	46.30
$MnCl_2,4H_2O$	1.81	9.10
$ZnSO_4,7H_2O$	0.22	0.80
$CuSO_4,5H_2O$	0.08	0.30
Na_2MoO_4	0.025	0.01
$CoCl_2,7H_2O$	0.025	0.01
Iron		
$FeSO_4,7H_2O$	10.008	370.00
Na_2EDTA	13.41	36.00

Frankia Media

MPN= MOPS + phosphate + nitrogen

4.2g MOPS buffer (mw 209.3)

1.7g K_2HPO_4

10mL NH_4Cl (0.5M stock)

bring up to 1000mL

pH to 7

Growing *Frankia* In a 500mL flask

Add 100mL MPN

Add carbon source

Autoclave

Add 1.7mL Trace Elements Mix

Add homogenized *Frankia*

Trace Elements Mix

20mL of 0.1M stock Na_2MoO_4

8mL of 0.5M stock $MgSO_4$

4mL of 20mM stock $FeCl_3$-NTA

2mL of 10X stock MOD-MBA trace salts

MOD-MBA trace salts

2.5g $Fe_2(SO_4)_3,7H_2O$

5g $MnCl_2,2H_2O$

0.25g $CuCl_2,2H_2O$

10g $CaCl_2,2H_2O$

0.5g H_3BO_3

1g $ZnSO_4,7H_2O$

0.2g $CoCl_2,6H_2O$

bring up to 1000mL in 0.1N HCl