Molecular Characterization of *Frankia* and Alder-*Frankia* Symbiosis in Eastern India

Thesis
Submitted in Fulfillment of the Degree of
Doctor of Philosophy

By

Balwinder Singh Bajwa

Supervisor

Dr. Arnab Sen

Department of Botany
University of North Bengal
Raja Rammohunpur, Darjeeling
West Bengal, India
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This work is dedicated to my beloved parents

Shri Jasbir Singh Bajwa

&

Smt. Manjit Kaur Bajwa

University *of* North Bengal
Department of Botany

Dr. Arnab Sen LECTURER

CERTIFICATE

I certify that the thesis entitled, "Molecular characterization of *Frankia* and alder-*Frankia* symbiosis in Eastern India" submitted by Mr. Balwinder Singh Bajwa for the award of PhD degree of the University of North Bengal, embodies the record of the original investigation carried by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of Doctor of Philosophy (Science) degree in Botany. The work has not been submitted for any degree of this or any other university and is in accordance with the rules and regulations of the University of North Bengal.

Arnab Sen

(Arnab Sen)
Lecturer

Date: 26/7/04.

Raja Rammohunpur Siliguri 734430 INDIA Phone:+91 353 2582106 FAX:+91 353 2581546

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[Balwinder Singh Bajwa]

Contents

CHAPTER 1

Introduction

Symbiotic association, a perfect example of division of labour between two organisms, the outcome of which may lead to endeavors like nitrogen fixation. Reduction of atmospheric N_2 to ammonia and its further assimilation into amino acids and other bio-molecules enables gaseous nitrogen to incorporate into life processes. As all organisms need Nitrogen to survive, nitrogen fixation is probably the second most important biochemical pathway after CO_2 fixation. However, the ability to fix nitrogen is found only in one biologic kingdom, the Prokaryota (Sprent & Sprent 1990). Thus, other organisms have exploited the ability of prokaryotes to fix nitrogen by establishing various types of interactions (Werner 1992). Cyanobacteria and plant-microbe symbioses are considered to be among the major milestones in evolution of life on Earth, bringing together the two most essential biochemical pathway, carbon fixation and nitrogen fixation. There occur two main types of symbioses between nitrogen-fixing bacteria and vascular plants: one between *Rhizobium* and leguminous plants, and the other between *Frankia* and actinorhizal plants (Wall 2000). A large number of woody dicotyledonous plants making symbiotic association with actinomycetes, belonging to the genus *Frankia* are called actinorhizal plants (*viz. Alnus nepalensis, Eleagnus pyriformis, Myrica nagi, Casuarina eqisetifolia, Coriaria nepalensis, and Hippophae sp.*etc).

The rhizobia-legume symbiosis involves more than 1700 plant species of the family Fabaceae (Leguminosae) distributed in three sub-families: Mimosoideae, Ceaesalpinoideae, and Papilionoideae (Wall 2000) with bacterial partners belonging to the family Rhizobiaceae *(Rhizobium, Azorhizobium, Sinorhizobium, Bradyrhizobium,* and *Mesorhizobium*) (Crespi & Galvez 2000). Unlike *Rhizobium*, *Frankia* form symbiosis with 25 different genera of Dicotyledonous mostly woody plants belonging to 8 families. These plants called actinorhizal plants comprise more than 220 species symbiotically associated with the filamentous actinomycetes *Frankia.* However, in all cases of *rhizobia*-legume or *Frankia*-actinorhizal symbiosis, a new plant organ, the nodule, is developed in which the bacteria proliferate, express the most vital enzyme, the nitrogenase and fix nitrogen in to ammonia. These compounds are then assimilated and transported to the rest of the plants (Hirsch 1992;

Pawlowski & Bisseling 1996; Franche et al. 1998). Various genes of both host and bacteria are involved in the process of nodulation and nitrogen fixation. In some cases shoot nodules are found but they may be adventitious roots associated with micro-symbiont to form nodule, very similar in construction with root nodules but are located above the soil. Distinctive features of actinorhiza in anatomy with legume nodules are: 1) legume nodules have an innermost infected tissue bounded by nodule parenchyma and peripheral vascular bundles, where as actinorhizal nodules are characterized by a central vascular bundle and peripheral infected tissue surrounded by cortical nodule parenchyma; 2) actinorhizal nodules are ontogenically related to roots whereas legume nodules bear shoot-like anatomy.

Most of the Actinorhizal plants are woody shrubs or trees and are perennial dicots, except for *Datisca*, which has herbaceous shoots. They have in common a tendency to grow in marginally fertile soils and they often serve as pioneer species early in successional plant community development (Schwintzer & Tjepkema, 1990). Actinorhizal plants are some of the first plant species to colonize the damaged environment caused by natural disaster such as landslides and volcanic eruptions or erosions (Burleigh & Dawson, 1991). Pollen distributions, determined from analysis of marine sediment cores, show that actinorhizal plants colonize de-glaciated soils during times of major climatic change (Heusser & shackleton, 1979).Once established on a site, actinorhizal plants can fix N_2 and add nitrogen to the soil in the form of leaf litter and dead root tissue. Actinorhizal plants, hence, build up soil organic matter and create a more favorable habitat for other plants and soil organisms (Cracker and Major 1955; Lawrence et al. 1967; Olff et al. 1993; Chapin et al. 1994; Aplet 1990). Since, these plants often flourish on marginal soil; they have current and impending application in reclaiming and conditioning soils, producing timber and pulp and performing as windbreak, ornamental and fuel wood plants, etc. Globally they have potential for integrating in to schemes for addressing issues of reforestation. The relationship between *Frankia* and host species contributes significantly to global nitrogen cycles. Actinorhizal plants provide nitrogen rich organic matter and are often fundamentals to the dynamics and biodiversity of terrestrial ecosystems. Actinorhizal

plants have potential relevance in increasing the soil fertility of nitrogen deficient sites and thus set foundations for a more abundant ecosystem (Benson & Silvester, 1993; Lawrence et al. 1967; Conrad et al.1985; Hibbs & Cromack 1990; Thilenius 1990). Examples of important early successional actinorhizal plants in the western U.S. include sweetgale (*Myrica gale)* and sitka alder (*Alnus viridis*) in coastal wetlands of Alaska (Thilenius 1990), numerous species of *Ceanothus* in chaparral, forest and mountain shrublands (Hickey end Leege 1970; Leege 1979; Conrad et al. 1985), the genus *Alnus* in the Pacific Northwest (Hibbs & Cromack 1990), and species of *Dryas* in arctic and alpine habitats in Alaska (Cracker & Major 1955, Lawrence et al. 1967). Alders and dryads were common colonizers of glacial till following the retreat of continental glaciations in the northern hemisphere (Ritchie 1987).

Actinorhizal plants have worldwide distribution. Most of them are temperate plants. However, some members of Casuarinaceae and Myricaceae are native to the tropical regions. Species of *Alnus* or *Elaegnus* are found in hilly areas of the tropics.

Actinorhizal plants, *Alnus nepalensis* in particular, prefers moist, cool climates with mean annual temperature of 13-26°C and mature trees are tolerant to frost. This plant can grow at high altitudes (up to 3000 m) in both temperate and subtropical regions, with annual rainfall 500 to 2500 mm and a dry season up to about 6 months long. It is the most droughts tolerant of the *Alnus* species but best growth is obtained in areas where the mean annual rainfall exceeds 800 mm and the relative humidity is higher than 70%. They prefer soils that are moist and well drained, but not waterlogged. They do not require high soil fertility but prefers permeable soils and does poorly on dry, exposed ridge tops.

Alnus nepalensis is native to Pakistan, eastern Nepal, Bhutan, northern India, south-western China, upper Myanmar and parts of Indochina. This plant has been introduced to various countries in Africa, Central America and South-East Asia. It has been included in species trials in Burundi (Brunck et al., 1990), in Uganda (Okorio et al., 1994), in the highlands of Java (1350 m altitude) (Rostiwati & Suriamihardja, 1987), and in agroforestry trials in Bolivia (Mahboubi et al., 1997; Baker & Schwintzer, 1990).

Alders are pioneer species favored by high light levels and exposed mineral soils; in addition, their ability to fix atmospheric nitrogen facilitates establishment on geologically young or disturbed sites with low levels of soil nitrogen (Harrington et al., 1994).

The identity of the actinorhizal root nodules endophytes as actinomycetes was established in 1964 when the electron microscopy revealed the prokaryotic structure of the microorganism in *Alnus glutinosa* and *Myrica serifera* root nodules (Becking *et al.* 1964). A more detailed description became available only in 1978, when Torrey group first successfully isolated *Frankia* strain (CpI1, now known as HFPCI1 from *Comptonia peregrina* nodules) in pure culture (Callaham et al. 1978). The field of research involving *Frankia* and actinorhizal plants has undergone rapid expansion with this event. Since, pure *Frankia* strains cultivatable *in vitro* are available; it is now feasible to apply the modern techniques of microbiology, physiology, biochemistry, molecular biology and genetics to this group of nitrogen fixing actinomycetes. .

The microsymbiont of actinorhizal plants was first referred to as *Frankia* in 1888 by Brunchorst and was later classified as an actinomycete after studies by Krebber in 1932 (Quispel 1990). *Frankia* belongs to the family Frankiaceae in the order Actinomycetales. The genus comprised of gram-positive to gram-variable strains (Lechevalier & Lechevalier 1990). *Frankia* differentiate into three cell types: vegetative hyphae, sporangia, and vesicles. These different cell types can be produced in pure culture, in planta, and presumably in soil. *Frankia* grows as a filamentous colony on agar plates and being micro-aerophilic to reluctantly aerophilic are cultured in liquid media (Lechevalier & Lechevalier 1990). In batch static cultures, the bacteria grow as threadlike submerged colonies without aerial or floating growth, and when grown under nitrogen limitation, form three characteristic cell types: filaments, vesicles, and multilocular sporangia (Akkermans & Hirsch 1997; Benson & Silvester 1993). Vegetative cells are generally poorly branched. Vesicles are the site of nitrogenase expression and nitrogen fixation (Huss-Danell and Bergman 1990; Tisa and Ensign 1987). They exclude oxygen, thereby protecting nitrogenase (Parsons et al., 1987) and exhibit a distinctive metabolism (Tisa 1998; Tisa and Ensign 1987; Tisa

and Ensign 1988). Vesicles are usually spherical in cultivated *Frankia,* whereas in nodules they often assume different shapes (spherical, elliptical, club-shaped etc.). Vesicles can also be septate or nonseptate. The third type of differentiated structure, the multilocular sporangia, is filled with spores, which can remain for long periods in dry soil as infective particles (Tortosa & Cusato 1991). On the basis of the presence or absence of sporangia within a root nodule, *Frankia* strains have been classified as either spore$^+$ or spore$^-$ (Schwintzer 1990). Spore$^+$ strains appear to be much more infective than spore$^-$ strains (Normand & Lalonde 1982); both have been characterized at the molecular level (Simonet et al. 1994). All three cell types can be found in the symbiotic state (Newcomb & Wood 1987) with few exceptions. Cultivated *Frankia* cells behave as heterotrophic aerobic bacteria with doubling times of 15 h, compared with 3 h for rhizobia. Nevertheless, the growth of *Frankia in planta* seems to be unrestricted, because timing of root infection, nodule development, and host cell infection are similar to those of rhizobia-legume nodules. Thus, the difficulties of growing *Frankia* in culture or isolating *Frankia* from some plant species reflect our limited knowledge of isolation and growth requirements.

Like other N_2 fixing microorganisms, members of actinorhizal genus *Frankia* can reduce atmospheric nitrogen with the help of nitrogenase enzyme. The site of nitrogenase is in the vesicle. In vesicles, protection against oxygen toxicity at a high partial oxygen pressure is provided by the build up of lipid containing layers at the surface (Parsons *et al.* 1987). Freeze fracturing technique has been used to demonstrate this unique feature (Harriot *et al.*1991; Parsons *et al.*1987). Actinorhizal nodules show an optimum nitrogen activity at around 20 K Pa of oxygen and significant inhibition above 25 K Pa of oxygen (Rosendahl; *et al.*1988; Silvester *et al.* 1988). *Frankia*e growing in artificial media produce the same general morphological structure that they produce *in planta, including* sparsely branched hyphae, vesicles and multilocular sporangia containing non-motile spores. Sporangiospores are unique among actinomycetes. They are surrounded by multiple membranous layers; those are visible by electron microscopy. Though, in actinorhizal nodules, hyphae and vesicles are the most prominent features, spores and sporangia of the endophyte have also

been detected in different species (Schwintzer 1990; van Dijk 1978; Van Dijk and Merkus, 1976). The anatomy of nodule tissue shows vesicles those, may be globose, pear shaped or elongated (Torrey, 1985). The vegetative phase of *Frankia* displays septate, branched, filamentous hyphae, producing a mycelial mat when grown on solid media (Benson and Silvester, 1993). The vegetative form is exhibited in pure culture, infection and proliferation within the plant and presumably saprophytic life within the soil.

Although *Frankiae* are exacting in their growth requirements, they do not need complex media to grow well. A simple salt solution supplemented with organic acid such as Pyruvate or Propionate as a carbon source often is sufficient since *Frankiae* can fix nitrogen *in vitro* (Lechevalier and Lechevalier, 1990).

Actinorhizal plants are strong competitors of legumes in respect to the amount of nitrogen they fix on global basis. Furthermore, the ability to dispense large quantities of infective and effective strains for various host plants of economic importance for forestry is also increasing the interest both in evaluating and optimizing various combinations of host strains and in understanding the involvement of both partners in order to manipulate the symbiotic system in future (Baker 1987).

However, the study of *Frankia* in pure culture is always a difficult criterion. The difficulties in studying *Frankia* in pure culture as highlighted by Benson and Schultz (1990) are primarily related to its pleiomorphic growth form and are magnified in ecological studies of *Frankia*. *Frankia* cannot be directly isolated from soil and counted on plates, because they are very slow growing and poor competitors for readily available carbon sources. There are no selective media available for *Frankia* till date. Even isolation from nodules is problematic because of the inability of *Frankia* strains to compete with other contaminating microorganism. Besides, we cannot discard the idea that some *Frankia* strains may be non-culturable.

Previously the heterogeneity among *Frankia* strains have been studied on the basis of total cellular protein and isozyme patterns (Benson and Hanna, 1983; Benson *et al.*, 1984; Gardes and Lalonde 1987; Gardes *et al.*, 1987). Benson and Hanna

(1983) performed a study on *Alnus incana* stand and they placed 43 nodule isolates into five groups based on one-dimensional SDS- polyacrylamide gel electrophoresis of total cell proteins.

Previous attempts were limited due to difficulties with growing enough cellular material for DNA extraction and also by the low efficiency of classical lysis techniques of *Frankia* cells. Later by exploiting certain enzymes like lysozyme in combination with a drastic extraction method the Lyon group tried a number of DNA isolation procedures. However, the use of achromopeptidase by Simonet et al. (1984) gave better results.

Previous work on *Frankia* (Reddell and Bowen, 1985; Sougoufara et al., 1992) revealed that host may play an important role than the microsymbiont during the commencement and subsequent development of symbiosis. Past experiments, pertaining to physiological data, point towards a direct involvement of the host, but suffer from lack of molecular evidence. Therefore further studies are required in this field.

Fortunately, now we have the molecular tools to detect and analyze *Frankia* in soil and in nodules without the need for culturing the bacteria. Polymerase chain reaction (PCR) techniques using primers specific to 16S rRNA genes, intergenic region of 16S-23S rRNA, intergenic region of *nifD-nifK* genes, or rep-PCR primers etc. have been applied to *Frankia* isolated from almost all actinorhizal plant genera (Benson et al. 1996; Clawson et al. 1998; Jamann et al. 1993; Jeong et al. 1999; Murry et al. 1997; Nalin et al. 1995; Nazaret et al. 1991; Normand et al. 1996). Sequence analysis of the PCR amplified 16S r-RNA gene has greatly facilitated the systematic, evolutionary and ecological studies of various microorganisms (Olsen *et al.* 1986; Woese *et al.*1987). This technique is particularly valuable for non-culturable microorganisms and has been extensively used for the elucidation of the phylogenetic relationships of various microorganisms (Giovannoni *et al.* 1990), including *Frankia* populations within the alder root nodules (Mirza *et al.* 1992; Nazaret *et al.* 1991; Nick *et al.* 1992). Defined molecular phylogeny groupings of *Frankia* are obvious from these studies (Clawson and Benson 1999; Mirza et al. 1994; Rouviere *et al.* 1996).

Objectives

The main objective of the present research work was to characterize and hence investigate phylogenetic relationships and best possible Alder-*Frankia* symbiosis of uncultured *Frankia* strains from the root nodules of *Alnus nepalensis*, native of Eastern Himalayas by using modern molecular biology techniques. Research on actinorhizal plant, *Alnus nepalensis*, was initiated due to its importance in reclaiming and nourishing soil, acting as source of timber and fuel-wood plant, preventing soil erosion and addressing the issues of polydenitrification globally.

Considering the present standing of *Frankia* and actinorhizal plants and critical gap areas in this field chiefly with respect to Sikkim and Sub- Himalayan West Bengal, the aims of the present study are:

- Collection of alder-*Frankia* germplasm from different parts of aforementioned regions.
- Isolation, purification and genetic screening of axenic cultures as well as nodular acquaintances of diverse *Frankia* strains from Sub-Himalayan West-Bengal and Sikkim.
- Substantiation of the integrity of the culture by PCR amplification of *Frankia* specific DNA sequences and physiological tests.
- Cloning of altered PCR amplicons for further studies.
- Learning the influence of host on nitrogen fixation rate of in-nodule *Frankia*.

CHAPTER 2

Review of Literature

2.1. History of *Frankia* research

Nitrogen is the mineral nutrient, essential to all living organisms, which most often limits plant production. Though gaseous atmosphere is 80% dinitrogen (N2), it cannot be utilized as such. This is because the triple bonded structure N≡N is extremely stable and chemically very non-reactive on account of the high-energy bond of the molecule. Nitrogen from the atmosphere can be fixed abiologically (industrial synthesis, ultraviolet irradiation, lightning) as well as biologically. Industrial synthesis contributes about 60 million tones of nitrogen per year for fertilizer uses whereas biological nitrogen fixation contributes about 100-175 million tones per year. Biological nitrogen fixation (BNF) accounts for 65% of nitrogen currently utilized in agriculture, and will be increasingly important in future crop productivity, especially for sustainable systems (Sen & Bajwa. 2002).

Biologically the ability to fix molecular nitrogen, occur, in a diverse groups of microorganisms that exist, either as free-living diazotrophs or in symbiotic associations with plants (Young 1992). As free-living diazotrophs only prokaryotes (bacteria, blue green algae and actinomycetes) and archaebacteria can convert dinitrogen to ammonia. Symbiotic nitrogen fixing bacteria include the cyanobacteria, the genera *Rhizobium,Bradyrhizobium, Azorhizobium, Sinorhizobium, Mesorhizobium and Frankia* (Paul and Clark 1996; Brock 2000). The root-nodule is the most important symbiotic association between nitrogen fixing microorganisms and higher plants (Akkermans and Houwers 1979; Dixon and Wheeler 1986).

Unfortunately, the study of root nodules of non-leguminous plants was shrouded in the mist of misconceptions for a long period. Between 1886 and 1888, Hellrigel and Wilfarth published two papers, which brought an end to a 10-year-old controversy about sources of nitrogen for growth of plants and opened up new frontiers of plant microbial sciences. They showed that legume nodules fixed atmospheric nitrogen. They also gave an idea about nodule inducing ferment, showed that it was more or less specific, was killed by moderate heat, and harmed by drought. Nitrogen fixed in a nodulated legume was not immediately available to the neighboring plants and that small quantities of combined nitrogen did not affect nodulation whereas larger amount was inhibitory

(Quispel 1988). Hellrigel and Wilfarth gave the idea of the difference between nitrogen users and nitrogen accumulators. Alders were initially pushed into the nitrogen-accumulating group. However, Hiltner proved that actinorhizal plants could utilize atmospheric nitrogen. In all cases of rhizobia-legume or *Frankia*-actinorhizal symbioses, a new plant organ is developed in which the bacteria differentiate, express the enzyme nitrogenase, and fix nitrogen into ammonia. These compounds are then assimilated and transported to the rest of the plant. Legumes and actinorhizal plants develop root nodules as a consequence of compatible plant-bacteria interactions through the switching on and off of genes in both genomes to establish a newly developed, shared structure. In some cases, so-called shoot nodules are formed (Prin *et al.* 1992), but these aerial nodules are modified adventitious roots, having the same structure as root nodules but are located above the soil or the stems.

Although both *Frankia*-actinorhizal plant and *Rhizobium*-legume symbioses have been known for many years to benefit soil fertility, the nature of these nitrogen-fixing symbioses and the microsymbionts involved were discovered at the end of 19th century (Quispel 1990). Various anatomical studies, cytological studies and Studies by Hellrigel and Wilfarth have been done to compare leguminous and actinorhizal microsymbionts. Actinorhizal and legume nodules can be easily distinguished at the anatomical level (Pawlowski and Bisseling 1996). Legume nodules have a central infected tissue surrounded by nodule parenchyma and peripheral vascular bundles, whereas actinorhizal nodules are characterized by a central vascular bundle and peripheral infected tissue surrounded by cortical nodule parenchyma. Legume nodules have been proposed to have a shoot like anatomy, whereas actinorhizal nodules are ontogenically related to roots. In another study the legume root nodule bacteria isolated by Beyerinck failed to infect the non-leguminous plants which led to the conclusion that the two microsymbionts are two different microorganisms.

Study of the actinomycetes induced nodule symbiosis has lagged behind research on legumes largely because of the repeated failure of attempts to isolate and grow the bacterial endosymbiont in pure culture by many workers. Although until 1978 isolation and cultivation of *Frankia* strains were generally considered to be impossible, since that

year Callaham *et al.* (1978) reported the first successful isolation of *Frankia* from the root nodules of *Comptonia peregrina* (L.). Later on Sarma *et al.* (1998) have described a more convenient method for isolation of *Frankia* by Calcium-alginate beads both for isolation of *Frankia* from nodules and generation of single spore cultures. Borthakur *et al.* (1996)&(1997) observed germination of spores into active colonies even when incubated in nitrogen free medium. To date, *Frankia* strains from a number of other actinorhizal species are available in pure culture (Lechevalier 1986; Benson and Silvester 1993) and their growth requirements have been optimized (Akkermans *et al.* 1983; Blom 1982; Meesters *et al.* 1985; Tjepkema *et al.* 1980).

2.2. *Frankia*- The microsymbiont

Frankia belongs to the family Frankiaceae of the order Actinomycetales. The genus name *Frankia* was proposed in 1886 by J.Brunchorst to honor his mentor, A.B Frank a Swiss microbiologist. Later, Krebber classified it as actinomycetes in 1932 (Quispel 1990). The genus *Frankia* is comprised of gram-positive and gram-variable actinomycetes (Lechevalier and Lechevalier 1990). The first cultured *Frankia*, isolated from *Alnus* root nodules was reported by Pommer (1959), but unfortunately the culture was lost. In 1978, the first successful isolation of *Frankia* was reported from *Comptonia peregrina* root nodules (Callaham *et al.* 1978), beginning a new era in actinorhizal symbiosis research (Quispel 1990).

2.2.1. Taxonomy of the genus *Frankia*

Following the successful propagation of the first isolate, more and more strains became available, and problems of nomenclature and taxonomy became a matter of concern.

Since Brunchorst and Frank considered the microorganism *Frankia* to be a fungus, the name was not generally used until Becking, in 1970, revived it and redefined the genus as consisting of members of the Actinomycetales in a new family, Frankiaceae (Becking 1970). Becking further proposed that 10 species be established (*Frankia alni*,

F.elaeagni, F.brunchorstii, F.discariae, F.casuarinae, F.ceanothi, F.coriariae, F.dryadis, F.purshiae, and F.cercocarpi) based on the source plant, and, since numerous attempts to obtain the organisms had failed, he described the members as "obligate symbiotic organisms". *Frankia* strains still constitute a separate family within the order Actinomycetales. In Bergey's Manual of Systematic Bacteriology, vol.4 (1989), they are included among "actinomycetes with multilocular sporangia" (Lechevalier & Lechevalier 1989). Other genera in this group include *Geodermatophilus* and *Dermatophilus*. Recent 16S rRNA sequence analyses of representatives of these genera have revealed a relatively close phylogenetic relationship between *Frankia* and *Geodermatophilus* but not *Dermatophilus*; an invalidly described isolate from the Black Sea called "*Blastococcus*" also clustered with *Frankia* (Hahn *et al.* 1989). Thus, future descriptions of the family Frankiaceae should include members of these genera. The family Dermatophilaceae will consequently contain only the genus *Dermatophilus* (Hahn *et al.* 1989). The taxonomy of *Frankia* is being reevaluated because of the uncertainty regarding species recognition and the confusion arising from trivial strain designations (Lechevalier 1983), the initial classification proposed by Becking (1970) has been replaced with a system of registry numbers. The availability of numerous strains (Baker, 1982) helped in the clarification of the relationships among various members in this unique family Frankiaceae. It should be noted that only one genus and no species were recognized until 1988. This absence of recognition of species within the genus *Frankia* was mainly due to vast variations in the various morphological and physiological characters normally used for taxonomic purposes.

Since 1978 (Callaham *et al.* 1978) an increasing number of *Frankia* strains have been isolated, and attempts have been made to classify the strains. So far all the strains have been classified within the genus *Frankia*. This taxon is characterized by:

a. The ability to nodulate plants;

b. The ability to fix nitrogen;

c. Its unique morphological properties, vesicles and sporangia;

d. The presence of sugar, 2-o-methyl-D-mannose (Mort *et al.* 1983);

e. The presence of cell wall type III and phospholipid content of type I of actinomycetes (Lechevalier and Lechevalier 1990);

f. A high G+C% in the range of 68- 72%

The characters employed in actinomycete taxonomy in the past have been the size and shape of sporangia or spores, and production of cellular or soluble pigment. For *Frankia* these characters are not considered sufficiently stable to be useful (Lechevalier 1984). According to Weber *et al.* (1988) the morphological characters are stable but too diverse for identification use. A combination of different observations (like Sporangial size, frequency and shape; pigment production; urease activity and acetate decarboxylation) can be used to separate some of the strains, but do not allow general identification of individual *Frankia* strains. It should be noted that, although the available *Frankia* isolates can belong to various host specificity groups, this character is not used in the actual because some *Frankia* strains can infect two host specificity groups, such as *Alnus* and *Elaeagnus* groups and because some non infective strains of *Frankia* are known to exist. As mentioned above one stable character is the presence of a unique sugar, identified as 2-O- methyl D-mannose, which is a unique trait of Frankiaceae family (Mort *et al.* 1983).

Related strains can also be distinguished on the basis of electrophoretic separation of isozyme. Gardes *et al.* (1987) characterized *Frankia* strains by electrophoretic separation of isozyme of eight different enzymes. They analyzed diaphorase, leucine aminopeptidase's, phosphoglucose isomerase, esterase, maltate dehydrogenase, phosphoglucomatase, and superoxide dismutase. The results obtained from this analysis were used to classify strains by numerical analysis based on pair-wise similarity coefficients, and further to propose the delineation of groups of strains.

On the basis of physiology two suprageneric or sub generic groups have been proposed (Lechevalier and Lechevalier 1989; Lechevalier and Lechevalier 1979). Main differences are shown in table 2.1(See table 2.1 at the end of this chapter).

The "constant" (relatively invariable for all taxa) regions of the 16S rRNA nucleotide sequences of actinomycetes are valuable for determination of the proper

placement of genera within the order Actinomycetales and for discerning the position of the order Actinomycetales in the prokaryote, while it is widely believed that the "hyper variable" regions may help in phylogenetic classification at the genus or species level (Harry *et al.* 1991; Mirza *et al.* 1992). In a different approach by Simonet *et al.* (1991) two different sets of primers were designed. One of which was a universal set designed for nine nitrogen fixing genera and the other set was specific for the *nif*H-*nif*D regions of *Frankia* spp. augmentation of different strains of *Frankia* with these two sets of primers resulted in fragments of the length specific for the genus. Similarly universal and specific primers targeted to 16S rRNA gene region also generated the fragments of the size characteristic of *Frankia* spp. and also of the closely related genus *Geodermatophilus* and were therefore presumably specific for members of the family Frankiaceae. Members of the genus *Geodermatophilus* and *Frankia* were further separated on the basis of intergenic sequence [IGS] the 16S and 23S rRNA genes by designing two additional primers. The amplification results showed that the 561bp fragment generated by the *Frankia* differed from the size of fragments generated from *Geodermatophilus* strains and the other microorganisms tested. Although this technique is quite promising, more strains have to be analyzed before general conclusion can be drawn.

2.2.2. Taxonomy of the species

The most critical part of *Frankia* taxonomy is its speciation. Main difficulties include (1) individual strains often nodulate plants from different plant orders (Benson and Silvester 1993), (2) strains from same genomic species have been isolated from members of different plant families (3) some strains failed to re-infect their source plants (Mirza *et al.* 1991) and (4) unpredictable nodulating abilities have been observed among the *Elaegnus* strains (An *et al.* 1985; Fernandez *et al.* 1989). Nevertheless, a considerable degree of host plant specificity does occur, with the result that the host plant origin and the ability to nodulate within certain host specificity groups are relevant but not determinative characteristics for strain identification or classification.

Arranging *Frankia* strains into phenotypically related groups have proven to be a difficult task. Classical physiological testing is of little use in grouping *Frankia* strains since they all grow slowly (doubling times of 15 to 48 h or more and the results often

vary depending on how long the strains are allowed to grow. Consequently, several other approaches have been taken with various degrees of success. Early attempts to correlate serological groups with host range or other phenotypic parameters were largely abandoned when such correlations proved more complex than was initially thought. (Baker *et al.* 1981; Lechevalier *et al.* 1983).

Techniques applied to resolve these problems are morphological differences, in planta sporangia formation (Normand and Lalonde 1982), phylogeny of carbohydrate uptake (Lechevalier *et al.*1983; Ganesh *et al.* 1994), utilization of various substrates and enzyme production (Shipton and Burggraaf 1982), serology (Baker *et al.*1981; Lechevalier *et al.*1983), host specificity (Bosco *et al.*1992) isozyme patterns (Maggia *et al.*1990), whole cell difference in the ribosomal sequences of genomic species of isolates of Elaeagnaceae etc.

Lalonde *et al.* proposed the acceptance of the species *F.alni* and *F.elaeagni* and created *F.alni* subsp. *pommerii* and *F.alni* subsp. *vandijkii* (Lalonde *et al.*1988). This proposal was based on the results of biochemical and physiological studies done on strains isolated mainly from members of the families Betulaceae, Myricaceae, Elaeagnaceae, and Casuarinaceae.

Fernandez *et al.* (1989) identified nine genomic species among 43 isolates examined. Three genomic species were found among the strains that infect *Alnus* species, five were found among strains that infect members of the family Elaeagnaceae, and one contained 11 strains isolated from members of the Casuarinaceae. Nine strains (four isolated from *Alnus* species, two isolated from members of the Elaeagnaceae, and three isolated from members of the Casuarinaceae) were not classified and may represent additional genomic species.

The largest group of *Alnus* infecting strains, genomic species 1, was first established as genogroup I by An *et al.* in their early DNA homology work (An *et al.*, 1985). They established genogroup I with seven isolates from *Alnus* species plus one from *Comptonia peregrina* and one from *Myrica pensylvanica*, with DNA from Arl49 (from *A. rubra*) as a reference. The coherence of this group has since been confirmed by numerous phenotypic and genetic analyses and thus has a valid claim to the species

epithet *F.alni* as described by *Lalonde et al.* (1988) with strain HFPCpI1 as the type strain.

The proposal that the subspecies *F.alni* subsp. *vandijkii* and *F.alni* subsp. *pommerii* be established has generated some confusion in the naming of strain. As Beyazova and Lechevalier (1992) have pointed out, a subspecies of *F.alni* should be *alni*. Also, strain ArgP5^{Ag} (ULQ0132105009), which is the type strain for the *F.alni* subsp. *Vandijkii* group of Lalonde *et al.* (1988), was in genomic species 3 in the analysis of Fernandez *et al.* (1989) and had only a 2% DNA homology with members of genomic species 1, which contained HFPCpI1. Therefore, the proposal that *vandijkii* be considered a subspecies of *F.alni* is problematical, since a subspecies should be genetically close to but phenotypically distinct from the type species (Wayne *et al.* 1987). Because of the results from the DNA-DNA reassociation studies, the definition of the subspecies *vandijkii* and *pommerii* needs to be reconciled with current knowledge. DNA-DNA reassociations have confirmed the diversity in the physiology, morphology, and biochemistry of Elaeagnus strains (An *et al.* 1985; Fernandez *et al.* 1989).

An *et al.* (1985) found no genogroup among 10 isolates (5 from *Elaeagnus* species, two from *Casuarina equisetifolia* and one each from *Ceanothus americanus*, *Purshia tridentate*, and *Alnus incana* subsp. *rugosa*) tested against DNA from EuI1a from *Eleagnus umbellate*) or ArI4. The 13 Elaeagnaceae strains tested by Fernandez *et al.* (1989) constituted their genomic species 4 through 8. Genomic species 4 had six members. Species 5 had three, and species 6, 7, and 8 had one each. Unfortunately, the type species proposed for *F. elaegnii*, strain SCN10a (ULQ190201001) (132), was not studied, so its relationship to other genomic species remains unknown. Strains originally isolated from members of the Casuarinaceae showed a surprising degree of similarity. Fernandez *et al.* found that out of the 11 strains tested (Fernandez *et al.*, 1989), 8 constituted genomic species 9, with percent reassociations ranging from 69 to 100%. The three strains that remained unclassified were "atypical", since they failed to reinfect members of the Elaeagnaceae.

Nazarat *et al.* (1991) developed a protocol based on the PCR amplification and sequencing of 16S ribosomal DNA sequence to measure phylogenetic relationships.

They examined nine genomic species created by Fernandez *et al*, earlier in 1989 and found that with one exception, strains belonging to same genomic species had identical sequences and that they differed from other genomic species

Another important method for understanding phylogeny of *Frankia* is restriction fragment length polymorphism (RFLP) of *nif* complexes (*nif*AB, *nif*K and *nif*H). The *nif* D-K DNA IGS region in *Frankia* spp. is larger and more variable than the *nif* H-D IGS region; thus the PCR-RFLP study of *nif* D-K IGS has proven to be more useful for distinguishing among closely related strains. Nazarat *et al.* (1989) found highly homologous results for infective isolates obtained from *Casuarina* spp. regardless of restriction enzyme used, whereas non-infective strains differed from first group and among themselves. Akimov and Dobritsa (1992) observed high level of DNA-DNA relatedness among both infective and non-infective isolates. Simonet *et al.* (1989) did RFLP analysis of 100 isolates from single alder stand using *nif*-HDK probes and they found similarity. When they used *nif*AB probes, five groups were found.

Jamann *et al.* (1993) employed PCR-RFLP technique. They amplified intergenic spacer (IGS) and a part of genes in *nif* cluster through PCR using two primers composed of sequences from conserved *nif*-D and *nif*-K regions flanking *nif* D-K IGS region. They cleaved the Amplicon with four different restriction enzymes and patterns obtained were used as fingerprints for typing *Frankia* strains. They also revealed that the DNA of *Frankia* isolates obtained from members of Elaeagnaceae and Casuarinaceae could be amplified to yield a product of about 1380bp, whereas the DNA of isolates obtained from *Alnus* spp. were amplified only weakly or were not amplified. When these products were digested with various restriction enzymes like *Hinf-I*, a characteristic pattern is obtained i.e. products digested with *Hinf-I* grouped together the entire test isolates *Mip-I* and *Scfr-I* resolved these organisms into the genomic spp. previously determined by DNA-DNA pairing.

Marry P. Lechevalier and her colleague K. Beyazova used a novel and simple method to tackle species problem. They used restriction enzymes with less number of restriction sites (rare cutters) to cut DNA into fragments of bigger molecular weights. Fragments were separated with pulse field electrophoresis. This method is called 'low

frequency restriction fragment analysis' (LFRFA). Findings of Beyazova and Lechevalier (1992) through LFRFA confirmed results of Nazarat *et al.* (1991) that type strain of *Frankia alni* subsp. *pomme* clusters at some distance from other *Frankia* genomic species isolated from *Alnus* spp. Each of these approaches has yielded phenotypic and in some cases genotypic subgroups within host specificity groups.

Not all approaches have been taken with classification in mind; some have been used primarily to assess the diversity of strains isolated from an individual plant species or of strains obtained from a limited geographical area (Benson *et al.* 1984; Bloom *et al.* 1989; Gardes and Lalonde 1987). Of the techniques used, only DNA-DNA homology and perhaps the most recently developed restriction fragment length polymorphism can unequivocally place an unknown strain within a genospecies. The other techniques yield well-defined groups independent of phylogenetic relationships, but the sensitivity of the methods remains unknown. The available biochemical, physiological, and phylogenetic information on certain well-studied groups of strains has rekindled the desire to establish *Frankia* species. To do so, it is desirable to satisfy the consensus that new genotype species be defined in phylogenetic terms on the basis of DNA sequence data (Wayne *et al.* 1987). Thus, DNA-DNA reassociation kinetics that approximate 70% or greater DNA relatedness, together with a 5°C or less δT_m between strains, would support placement of two strains in the same species, with the caveat that any genospecies so identified should not be named until it is distinguishable from another genospecies on the basis of phenotypic properties. For *Frankia* strains, sufficient information is available to support the species identification of some members of the genus.

2.3. Actinorhizal plants-The host

The plants nodulated by *Frankia* are known as actinorhizal plants (Torrey, J.G. and J.D. Tjepkema 1979.). According to Benson and Silvester (1993), there are 194 species of 24 genera, but there is no detailed account given. Till date about 288 species of 24 genera belonging to 8 families of 7 orders have been reported to have actinorhizal associations (Baker and Schwintzer 1990), some of the important *Frankia*-actinorhizal

symbiosis can be seen in (Plate I & II). Table-2.2(See table 2.2 at the end of this chapter) shows some important symbiotic associations between *Frankia* and actinorhizal genera.

Actinorhizal genera are found in ancient as well as advanced lineage. They like to grow in marginally fertile soil and can be found in most of the climatic zones inhabiting a variety of ecosystems including arctic tundra (*Dryas* species), coastal dunes *(Casuarina, Hippophae, Myrica,* and *Elaeagnus* species), riparian (*Alnus* and *Myrica* species), glacial till *(Alnus* and *Dryas* species), forest (*Alnus, Casuarina, Coriaria,* and *Shepherdia* species), chapparal and xeric (*Casuarina, Purshia, Ceanothus, Cercocarpus, Comptonia, and Cowania* species), and alpine (*Alnus* species).(Benson and Silvester 1993).

Actinorhizal plants are distributed worldwide and occupy a wide range of habitat. They are native to Pakistan, eastern Nepal, Bhutan, northern India, southwestern China, upper Myanmar and parts of Indochina. They are introduced to various countries in Africa, Central America and South-East Asia. Table 2.3 shows the distribution of native actinorhizal families and genera (See table 2.3 at the end of this chapter).

Distribution of Actinorhizal plants in India is shown in Fig.2.1 (See figure 2.1 at the end of this chapter)

2.3.1. Taxonomy of host plant

All the actinorhizal plants are trees or shrubs, except for the genus *Datisca,* which is herbaceous. Besides, all the members of a certain family may not be actinorhizal, while all the genera of the family Elaeagnaceae are actinorhizal. They cover a range of woody dicotyledons and the taxonomic relation is very less. Some species are very well adapted to flooded lands, warm arid and semiarid regions and areas of devastation (for example, rock slides). The taxonomic position of actinorhizal genera in the Magnoliopsida according to Cronquist (1988) is quite different from the position in Hutchinson's (1973) classification .Particularly, in Cronquist's classification, Rosales are not ascribed to a position anterior to the Hamamelidales and the Elaeagnaceae and Rhamnaceae are found in different orders and are shown to have diverged from the

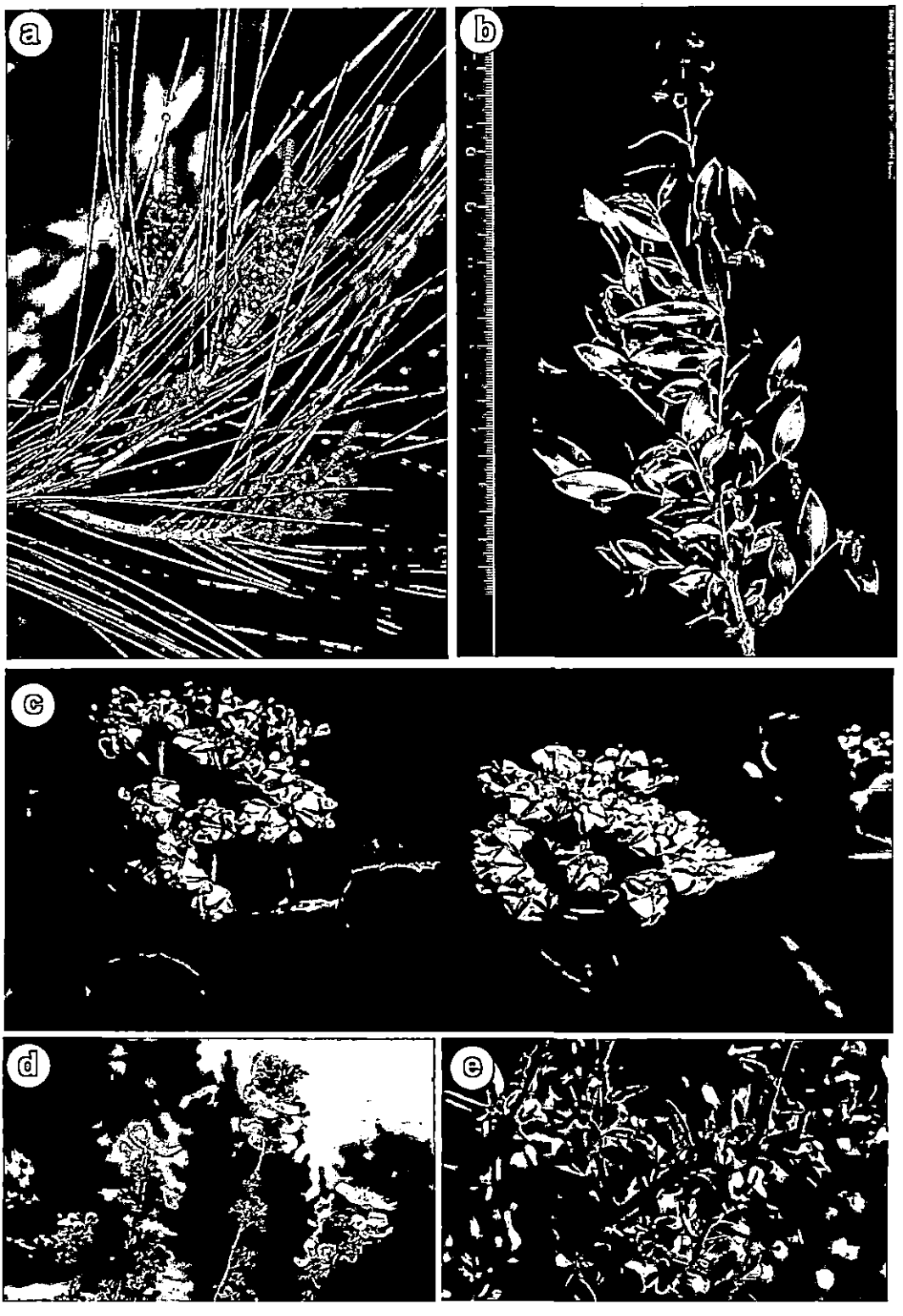

Plate I

Representatives of Actinorhizal plants : (a) A twig showing inflorescence of *Casuarina glauca* (b) A flowering twig of *Coriaria myrtifolia* L. (c) Flowering twig of *Ceanothus gloriosus* var. *Exaltatus.* (d) An inflorescence of *Pueshia stansburiana* (e) A plant of *Cercocarpus* sp.

Photo courtesy : www.ibiblio.org.; www.Herbarl Virtual, Universitat Illes Balears; www.nazflora.org.

Plate II

Representatives of Actinorhizal plants : (a) Flowers of *Cowania* sp. (b) A plant of *Cowania* sp. (c) Plant with fruiting body of *Eleagnus multiflora* (d) A plant of *Myrica gale* showing its flowers.

Photo courtesy : www.unps.org; www.floridata.com.

Rosales. Datiscaceae are found in the Violales (Dilleniidae and Casuarinales are grouped together with Fagales and Myricales in the Hamamelidae.Pollen records show, members of the orders Fagales and Myricales were the first among the actinorhizal plants to visit the earth. Rosales, Proteales and Rhamnales are relatively advanced.

The host involved in the present study is *Alnus nepalensis*, commonly called alder. The alders belong to the family Betulaceae. They are known by various Synonyms such as *Alnus mairei*, *A. Léveillé*, *Clethropsis nepalensis* Spach. The vernacular/common names of alder are Utis (Nepal); maibau (Burma); piak (India); meng-zi-qi-mu, han-dong-gua (China); tong quan su, tong qua mu, tong po mu (Viet.);Indian alder, Nepalese alder (Eng.) (Joker 2000).

Alnus prefers moist, cool climates with mean annual temperature of 13-26°C and mature trees are tolerant to frost. It can grow at high altitudes (up to 3000 m) in both temperate and subtropical regions, with annual rainfall 500 to 2500 mm and a dry season up to about 6 months long. *Alnus* species are mostly drought tolerant but best growth is obtained in areas where the mean annual rainfall exceeds 800 mm and the relative humidity is higher than 70%. They prefer soils that are moist and well drained, but not waterlogged. *Alnus* does not require high soil fertility but prefers permeable soils. It does poorly on dry, exposed ridge tops (Joker 2000).

The alder plants are deciduous or semi-deciduous trees, typically about 30 m tall and with a diameter of 60 cm but on good sites it can reach 35 m and a diameter of 2 m. Leaves are simple, alternate, 6-20 cm long, slightly serrate with prominent parallel veins. The flowers are unisexual, female and male flowers in separate inflorescences called catkins. Male catkins 10-25 cm long, are drooping, in terminal panicles. Female catkins 1-2 cm long, 3-8 together in axillary racemes. The fruiting catkins resemble cones. They are dark brown, 1.5-2 cm long, upright on short stalks, elliptical and with woody scales. The empty catkins persist on the tree. The seeds are light brown, circular, flat nut, with membranous wing, more than 2 mm across. Eight kg of catkins contain about one kg of seed. There are typically 2.3-3.5 million clean seeds/kg. (Joker 2000).

In Nepal, seed is collected between November and March depending on locality. In Yunnan (China) seed ripens in December. The catkins are collected directly from the

tree when they turn yellowish-brown and begin to open, but before the seeds have been dispersed by the wind. Catkins from previous years can persist on the tree; they are dark brown or black, contain no seeds and should be avoided. (Joker 2000).

2.4. Actinomycetes symbiosis

Frankia forms symbiotic association with botanically diverse group woody shrubs and small trees belonging to well over 200 species. Actinorhizal plants are classified in 24 genera, eight families and seven orders as mentioned in section 2.3. Generally all species belonging to these 24 genera are able to form root nodules under proper conditions. Few exceptions however exist in Rosaceae. Some of the genera with nodule bearing species, e.g. *Casuarina*, *Coriaria* and *Myrica* have an isolated taxonomic position and are classified in orders with only one genus. In other cases the taxonomic position of nodulated species may vary considerably. For example, in Fagales, only one genus, i.e. *Alnus* is nodulated, while other genera of Betulaceae and other families of this order are non-nodulated. In Rhamnales, nodulated genera are only known in only two families, viz. Elaeagnaceae and Rhamnaceae. In Elaeagnaceae nodulation is the characteristic feature of whole taxon. In Rhamnaceae, however, only three genera viz. *Ceanothus*, *Discaria* and *Colletia* have been reported to be nodulated. However, only a few species of 16 of the 58 genera of this family have been searched for nodulation (Bond 1976). Hence there remains a probability of finding a new nodulated species in this family. If nodulation were considered to be genetically determined, one would expect that genera taxonomically related to nodulated genera, also includes nodulated species.

The strong *Frankia*-actinorhizal association reveals that nodulation does occur in various taxa of host plants, which are not always taxonomically related. However, within the taxa, nodulation in general is a feature of the genus, and often even of the whole family. Therefore plant taxonomy is an important key in discovering nodulated plants. An additional key in this study is ecology. Most nodulated plants grow on nitrogen poor soils. Such conditions are favorable to either plants with nitrogen fixing root nodules, or

to plants, which grow oligotrophically. Hence the importance of systematic study of the occurrence of nitrogen fixing root nodules cannot be ruled out.

In order to determine the efficiency of nitrogen fixation Huss- Dannel, 1990 made a thorough assay on the nodule respiration, nitrogenase activity and hydrogenase activity. With one exception of the "local source" of *Frankia* from Sweden (Sellstedt and Huss-Danell 1984; Sellstedt 1989), hydrogenase activity is universally present in *Frankia* and actinorhizal nodules. Hence nitrogenase activity undoubtedly is the strong criteria in characterizing the host-*Frankia* symbiosis. Some *Frankia* strains are infective but ineffective i.e. they are capable of forming nodules but do not fix nitrogen. Ineffective nodules are typically small in size, contain relatively a few *Frankia*, and do not normally contain vesicles (Hahn *et al.* 1988; Berry and Sunell 1990). Ineffective nodules often occur because of intergeneric and interspecific incompatibilities (Weber *et al.* 1987; van Dijk *et al.* 1988); however some *Frankia* isolates form ineffective nodules even when inoculated on the same host species from which they are isolated (Hahn *et al.* 1988). Van Dijk and Sluimer-Stolk (1990) report that some ineffective *Frankia* have no nitrogenase actvity in pure culture and their DNA does not hybridize with *nif-HDK* probe. Hahn *et al.* (1989) found significant differences in the 16S rDNA sequence of an ineffective compared to an effective *Frankia* strain. In some cases effectiveness can be lost by mutations (Faure-Raynaud *et al.* 1990).

2.4.1. Role of host in symbiosis

The actinomycete symbiosis with the host plant suggests that the host cells and tissues exert a remarkable degree of control over the expression of the invading microorganism. This host control is part of the symbiotic relationship which keeps the invader under control and allows the symbiosis to work to the benefit of the host while providing the endophytic symbiont a site and substrates to function.

The nitrogen fixing effectiveness of host *Frankia* species have been studied primarily with *Casuarina* and *Alnus* species. Over three fold differences in growth and nitrogen fixation have been found for various *Casuarina-Frankia* combinations (Redell and Bowen 1985; Fleming *et al.* 1988; Redell *et al.* 1988; Sanginga *et al.* 1990), although

Rosbrook and Bowen (1987) found no significant *Frankia* strain effect. Differences in host provenance may be greater determinants of growth and nitrogen fixing effectiveness than variations among *Frankia* strains (Sougoufara *et al.* 1992). Similar differences in growth and nitrogen fixation have been found for *Alnus-Frankia* combinations (Normand and Lalonde 1982; Wheeler *et al.* 1986; Hooker and Wheeler 1987; Weber *et al.* 1989). In *Alnus-Frankia* symbiosis, greater nitrogen fixing effectiveness has been reported for Sp^- compared to sp^+ symbioses (Normand and Lalonde 1982; Wheeler *et al.* 1986), although this relationship is not always observed (Kurdali *et al.* 1990). Despite the large variation in nitrogen fixing capacity of various host-*Frankia* combinations, no clear superior host genotype or *Frankia* strain have been recognized and any positive effect of inoculation with superior *Frankia* strain may soon be overshadowed in the field by indigenous *Frankia* (Hooker and Wheeler. 1987). In *Alnus-Frankia* symbiosis, it has been shown (Hall *et al.* 1979) that an important improvement in the rate of nitrogen fixation can be gained by the selection of host plant, which is more active in photosynthesis, the key element for nitrogen fixation activity. Furthermore the susceptibility of the host plant to a *Frankia* strain is variable and consequently selectable (Tremblay *et al.* 1984).

There is remarkable difference in the rate of symbiosis in terms of ultra-structure of *Frankia* in pure culture. The host genotype is shown to control the nodule morphology. The symbiosis in *Myrica gale* and *Comptonia* may represent an intermediate situation between *Alnus* and *Casuarina*. The terminal vesicle within the root nodule may vary in shape considerably from overtly enlarged club shaped terminal endings to only slightly enlarged elongate terminal filaments. The state of vesicle envelope within these nodules has not been determined. As in *Casuarina*, the cell walls of infected *Myrica* nodules show specialization (VandenBosch and Torrey 1985) and intermediate level of haemoglobin like compound has been reported (Tjepkema 1983). Atmospheric levels of O_2 are available within the nodule to the infected cell (Tjepkema 1983) and even in water logged sites, aeration of the nodules through air passages within the nodule roots allows the nodules to undergo aerobic respiration and would necessitate some special modifications of the infected cells to achieve O_2 protection of the

nitrogenase within the *Frankia* filaments and vesicles. The interesting and peculiar orientation of the elongate vesicles in *Datisca* and *Coriaria* suggests yet another modification, which may have evolved to achieve the end of O_2 protection of the endophyte nitrogenase.

2.4.2. *Alnus-Frankia* versus legume-*Rhizobium* symbiosis

Although rhizobial symbiosis is by far the best studied of the nitrogen-fixing symbiotic associations with higher plants, many others exist. Symbiosis between woody dicotyledonous plants and the actinomycetes, *Frankia* (a filamentous prokaryote), has been reported in 21 genera covering eight different families and is believed to have a role in nitrogen-fixation comparable to that of rhizobia symbiosis.

Many similarities exist between rhizobial and the *Frankia* symbiosis. The mode of symbiosis and root nodule induction procedure of *Frankia* and *Rhizobium* are more or less similar, both lead to the formation of nodules on the roots of the host plant in which nitrogen fixation occurs. In both cases there is a complementarily with some of the *nif* gene sequences of *K. pneumoniae* and a number of associated proteins are related. However, *Frankia* doesn't invade the host cell and is not therefore surrounded by a poribacterial membrane. Instead it is encapsulated in carbohydrate material derived from the host plant. This may not be distinctive since legume (*Andria* sp.) has shown that similar encapsulation occurs. Other evidences suggest that the *Frankia* symbiosis involves hemoglobin similar to that found in legumes and that the mechanism regulating expression of nitrogenase in presence of oxygen and ammonium are very similar in both *Rhizobium* and *Frankia*. They differ markedly in development in morphology and development. Infected zone of legume nodule is central and is normally contending within an endodermis and an inner cortical layer of tightly packed cells. Actinorhizal nodules normally have a central stele that has infected tissue adjacent to it or around it (Benson and Silvester 1993).

2.4.3. Importance of Alder-*Frankia* symbiosis

Study of the *Frankia* actinorhizal symbiosis is important for several reasons, as a pioneer, nitrogen-fixing species alders are suitable for soil improvement and rehabilitation of degraded lands, including the use of actinorhizal shrubs and trees for reforestation and reclamation of depauperate, nitrogen limiting soils. Seeds have been broadcast to stabilize landslides (Gordon and Wheeler 1983). In Burma, it has been used with success to reforest abandoned taungya areas. In agro- forestry systems, it can be interplanted with a number of crops. The wood is an important source of firewood and charcoal.

For example, as more and more land, especially in the tropics, becomes deforested, the utilization of fast growing, nitrogen fixing trees for reforestation purposes becomes critical. According to the Office of the Technology Assessment (OTA) (1984), about 11.3 million hectares of what remains of tropical forests are destroyed annually. In addition many non-leguminous, nitrogen-fixing plants are colonizers of poor soils. After the Pleistocene glaciation's, plants such *as Alnus, Dryas, Elaeagnus, Hippophae*, and *Shepherdia* played an important role in soil reconstruction. (Lawrence *et al.* 1967). Currently their role in nitrogen fixation is recognised as being at least comparable to that *Rhizobium*-legume symbiosis and attempts are being made to utilize actinorhizal trees in reforestation and soil reclamation in developing countries. Besides the practical applications of actinorhizal plants to situations described above, the study of the symbiosis offers much interest to those investigating the basic biology of nitrogen fixing symbiosis. There are a number of parallels, developmental and genetic, between the *Frankia*-nonlegume symbiosis and the *Rhizobium*-legume association, which make the former interesting to study in terms of molecular biology. Some of these similarities are striking. For example, it has been known for some time that the *nif* genes of *Klebsiella pneumoniae* hybridize with DNA sequences from *Frankia* as well as from *Rhizobium*. Both the *Rhizobium*-legume and *Frankia* -nonlegume symbiotic associations lead to the formation of root nodules in which the microbes fix atmospheric nitrogen to ammonia. However, we must keep in mind not only the similarities, but also the differences, between the two types of symbiosis.

In exchange for a water and nutrient supply, the bacteria enable alders to fix much needed atmospheric nitrogen, which in turn enriches the surrounding soils. This increases the fertility of the soil for many species and makes alders as important species in ecological successions, land reclamation and remediation. Thus, *Frankia* have a direct and important impact on the environment and it makes the alders as an important and unique species in the world.

2.5. *Frankia* Physiology

The hydrogen metabolism of *Frankia* seems to be an interesting physiological characteristic. All nitrogen-fixing organisms evolve hydrogen from the nitrogen-fixing enzyme, nitrogenase, when nitrogen is reduced to ammonia. This means that some of the energy used in the nitrogenase reaction is wasted on nitrogen evolution. Root nodules of many legumes (*Rhizobium* symbioses) evolve considerable amounts of hydrogen with as much as 30%-60% of the electron flow through lost as hydrogen (Schubert and Evans 1976) .In contrast, *Frankia* symbioses were claimed to have little or no net hydrogen evolution (Moore 1964; Schubert & Evans 1976, Roelofsen & Akkermans 1979). In some strains of *Rhizobium* and *Frankia* hydrogenase is present (Roelofsen and Akkermans 1979; Benson *et al.* 1980) and recycles (part of) the hydrogen evolved from nitrogenase. Hereby some energy is regained and no or little net hydrogen evolution is measured.

Recently lack of hydrogenase activity was reported from a symbiosis between *Frankia* and *Alnus incana* (Sellstedt and Huss-Danell 1984). This *Frankia* strain makes it possible to study the importance of differences in hydrogen metabolism for growth and nitrogen accumulation in *Alnus*. The purpose of the present study was, therefore, to compare symbioses between one clone of *Alnus incana* and different strains of *Frankia* with respect to nitrogen fixation and growth of alders in relation to hydrogen metabolism of the *Frankia* strains

2.6. *Frankia* in pure culture

2.6.1. Morphology of *Frankia* in pure culture

Invitro studies of *Frankia* were possible only after the isolation and subsequent axenic culture on yeast extract medium. But improvements in the composition of culture media now permitted *Frankia* to be grown *invitro* much faster than earlier used yeast-extract medium (Lalonde and Calvert, 1979). In their classical reviews Newcomb and Wood (1987) and Benson and Silvester (1993) gave vivid descriptions of structure and ultra structure of *Frankia* cells. Benson and Silvester (1993) also discussed the difficulties of its study with electron microscope because of the delicate nature of some components of cells.

In pure culture *Frankia* behave as micro-aerophilic and mesophilic microorganism (Burggraaf and Shipton, 1982). It forms dense mat of anastomosing hyphae with sporangia developing terminally and at intercalary positions when grows *invitro* (Newcomb and Wood, 1987). Unlike other symbiotic nitrogen-fixing bacteria, *Frankia* is multicellular and differentiated. During the life cycle, vegetative hyphae, vesicles and sporangia develop in response to partially defined conditions (nitrogen limited). *Frankia* strains are characterized by the presence of vesicles and hyphae bearing large sporangia when cultivated under proper conditions. These spherical structures have not yet been found in other kind of actinomycetes and can be utilized as the feature of the genus *Frankia*. However, spore formation and induction of vesicles are largely dependent on the culture conditions. This makes the identification of the strain difficult. In many strains the production of vesicle is suppressed by the presence of combined nitrogen in the medium, i.e. conditions which also repress the nitrogenase.

2.6.1.1. Hyphae

Frankia strains are readily recognized by their appearance in liquid culture. They form extensive hyphae and spores on multilocular sporangia located either terminally or in intercalary position on the hyphae. Aerial hyphae are not produced on solid media. Free-living cells under the light microscope show branched sepate hyphae ranging in

width from 0.5 – 1.5 μm in diameter. The hyphal cell wall is composed of two layers of electron dense material, a base layer and an outer layer. Base layer gives rise to cross walls. Internally hyphal cells contain numerous rosette- shaped granules, which are presumed to be glycogen and lipid droplets. An extra cellular multilayered envelope was first identified in hyphae of free-living *Frankia alni* HFPCpI1 (Newcomb *et al.* 1979).

2.6.1.2. Sporangia

Frankia sporangia are observed in the cortical tissue of some actinorhizal nodules. Sporangia in the nodule structurally resemble those formed in free-living culture, and are located both within infected cortical cells and in intercellular spaces of some hosts. In *Alnus*, *Myrica* and *Comptonia*, differentiation of sporangia coincides with vesicle senescence (VandenBosch and Torrey 1984). Host cytoplasm degenerates in cells where sporangia are present.

Frankia sporulate spontaneously in submerged culture, forming multilocular sporangia, which are round, cylindrical or irregular in shape (Newcomb *et al.* 1979). Sporangia develop as terminal or intercalary structures and remain filled with thick walled, refractile and non-motile spores that are roughly geometrical in shape (Newcomb and Wood, 1987). The mature spores show evenly dispersed, but the tubules, which are such a prominent feature of hyphae, are not present in the developed spores (Lancelle *et al.* 1985). Cytoplasm Spores are surrounded by a lamina and their cytoplasmic content is similar to that of the hyphae. The tubules, which are present in the laminate envelopes of hyphae, are absent in the sporangia (Lancelle *et al.* 1985). Sporulation in nodules is well documented only for *Alnus* and *Myrica* species and even in these, is sporadic and somewhat site specific (Torrey 1987). Sporulation in nodules of other genera is not well documented. Strains, which release spores, show good germination in culture (Tzean *et al.* 1989) and, coincidentally perhaps, also show low levels of sporulation in host plants (Racette *et al.* 1991)

Although virtually all *Frankia* can be induced to form sporangia in pure culture, this is not necessarily the case in planta. Van Dijk and Merkus, (1976) observed that

Frankia within actinorhizal root nodules either forms many sporangia (Sp+) or no sporangia (Sp-). Smolander and Sundman (1987) proposed an intermediate class for nodules devoid of sporangia, however, Schwintzer (1990) suggests that intermediate nodules are better classified as Sp-.The spore type of actinorhizal nodules is of interest for at least three reasons: (1) It is doubtful if any *Frankia* have been isolated from Sp+ nodules, (2) Interesting and complex ecological relationships have been observed between Sp+ and Sp- strains of *Alnus* sp. and *Myrica* gale (Weber 1986; Holman and Schwintzer 1987; Kashanski and Schwintzer 1987; Smolander and Sundman 1987), and (3) There is some evidence that Sp+ and Sp- nodules differ in both absolute and relative nitrogenase efficiency

2.6.1.3. Vesicles

Frankia produces vesicles *in vivo* (exception: *Casuarina*). Most isolates of *Frankia* differentiate vesicles at the end of hyphal tips under conditions of ammonia starvation (Fontaine *et al.* 1984). In host plants, *Frankia* vesicles assume various shapes. In nodules of *Alnus* and *Elaeagnus* the spherical vesicles are arrayed around the periphery of infected cortical cells of the nodule lobes (Lalonde and Knowles 1975; Baker *et al.* 1980). Vesicles are internally septate, stiptate and circular or cone like in shape; approximately 1.5-2.0µm in diameter. Vesicles are mainly of three types: (1) Spherical vesicles are found in *Alnus* and *Elaeagnus* nodules, (2) Members of Rosaceae i.e. *Cercocarpus* and *Dryas* species have nonseptate and elliptical vesicle (nitrogenase protection mechanism is unclear in this case), (3) Club shaped hyphal endings which can be called as vesicles are found in the species of *Casuarina* and *Comptonia* (Newcomb and Wood 1987).

Vesicles originate as basally septate, stiptate swellings of hyphae or short side branches, termed pro-vesicles. The densely cytoplasmic pro-vesicles often show evidence of internal septation. The most important physiological function of vesicles is to shelter nitrogenase, the key enzyme involved in nitrogen fixation. As in all prokaryotes the enzyme nitrogenase in *Frankia* is oxygen labile (Benson *et al.* 1979). Within the vesicle, nitrogenase is protected from deleterious effects of oxygen. Special protection of

the enzyme is provided in a variety of ways. One hypothesis is that the multilaminate vesicle envelope developed in response to increase in ambient pO_2 provides a physical barrier to inward diffusion of oxygen, thereby protecting nitrogenase within the vesicle from denaturation by O_2.

Vesicles are also found in planta, where their shape or presence can be determined by the host (Baker and Mullin 1992). Vesicles are notably absent from nodules of species of *Casuarina* and *Allocasuarina*, yet isolates from these nodules differentiate vesicles in culture (Tjepkema and Murry 1989). Since in *Casuarina* nitrogen fixation occurs in absence of *Frankia* vesicles, a specialized host cell wall with hydrophobic properties, which could function as oxygen diffusion barrier (Berg and McDowell 1988), is thought to be the structural barrier for nitrogenase.

Vesicles have been proposed as the sites of nitrogen fixation (Noridge and Benson 1986; Meesters 1987). In most *Frankia* strains vesicles are formed only in response to nitrogen limitation (Fontaine *et al.* 1984; Murry *et al.* 1984; Tjepkema *et al.* 1980), but some strains can produce vesicles even on nitrogen rich media (St.Laurent and Lalonde 1987; Meesters 1987; Tisa *et al.* 1983).

2.7. Nodule development

Actinorhizal root nodules are established through a series of interactions between *Frankia* and host root cells. The nodule is system of structurally and developmentally modified lateral roots. The structural changes observed in the development of nodules reflect the molecular events in which normal patterns of plant development are altered.

2.7.1. Nodule morphology

Actinorhizal root nodules are perennial, coralloid structures consisting of multiple nodule lobes. Field collected nodules 3-5 cm. In diameter are not uncommon. Nodules may consist of discrete lobes, as in *Myrica* or *Ceanothus*, or the lobes may be densely packed as in *Alnus*. Each nodule lobe is a modified lateral root. The nodule meristem gives rise to mature tissues, including a vascular cylinder, cortical tissue and superficial

periderm. In most cases, nodule lobe growth is determinate. *Frankia* infects only cells of nodule cortex.

In several actinorhizal hosts, a more indeterminate pattern of growth is reflected n the formation of nodule roots from the apex of mature nodule lobes. In *Myrica* and *Casuarina*, nodule roots, which are negatively geotropic, develop from most nodule lobes. Nodule roots are devoid of *Frankia* hyphae and vesicles, and so do not contribute to nitrogen fixation. Nodule roots often contain large air spaces, an anatomical feature that facilitates oxygen diffusion.

2.7.2. Conditions for infection

.The infection process begins in the rhizosphere. Conditions necessary for colonization and initial binding of *Frankia* to the host root have not been entirely elucidated. *Frankia* hyphae are embedded within a mucilage layer at the root hair or epidermal surface. This mucilage layer is evidently secreted by the root hairs (Berry and Torrey 1983), although the root cap also produces mucilage. Elements of the host-derived matrix may participate in attachment phenomenon.

In case of root hair infection, though one infection is enough to bring about nodulation, frequency of nodule formation is directly proportional to amount of inocula (Newcomb and Wood 1987). More than one strain has been isolated from single nodule. It implies that more than one infected root hair sometimes forms one root nodule

As long as the conditions favorable for root growth are present, environmental factors such as pH or nutrient availability do not appear to limit infection and nodulation, at least for *Frankia* strains isolated to date. "Helper" microorganisms for example, *Pseudomonas* sp., improve nodulation of both *Alnus* and *Casuarina* under controlled growth conditions (Knowlton *et al.* 1980). The effect of these organisms appear to be two fold, first in including root hair deformation, which may involve altered cell wall deposition, and second in increasing the production of root hair extra cellular matrix. Even under favorable rhizosphere conditions, infection is not assured. Compatibility factors, about which little is known influence the potential for successful infection and nodulation.

2.7.3. Infection Pathways

Frankia infects its host plant in two different ways. In case of *Alnus, Casuarina Comptonia, Myrica* etc., infection takes place through root hair deformation (Berry and Torrey 1983; Callaham *et al.* 1979; Torrey 1976), while for other hosts like *Ceanothus, Elaeagnus, Shepherdia,* etc.; early colonization of root tissues by *Frankia* is entirely intercellular (Miller and Baker 1986; Racette and Torrey 1989). Despite these differences in early stages of cellular interactions between host and microsymbiont, eventually *Frankia* penetrate host cell walls in developing nodule lobes and differentiate within the cells. The same *Frankia* strain can, in some instances, infect different hosts by different pathways (Miller and Baker 1986; Racette and Torrey 1989). These observations indicate a common genetic basis for infection.

2.7.4. Infection Process

2.7.4.1. Root hair deformation

Frankia can take the help of other soil bacteria in deforming host plant root hair. Knowlton *et al.* (1980), Berry and Torrey (1983), reported that in case of *Alnus rubra,* deformation of root hair occurs rather quickly (within few hours) with a helper bacterium such as *Pseudomonas capacia.* Presence of helper bacteria also increases the rate of nodulation, though its presence is not compulsory for the process.

Root hair infection has been described in *Myrica, Comptonia, Alnus and Casuarina* (Torrey 1976; Berry *et al.* 1986). This process involves excessive changes in host cells beginning with wall deformation of expanding root hairs. Growing root hairs are perturbation by rhizosphere bacteria, especially by *Frankia.* Responding to unknown signals, growing root hairs initiate multiple tips, and tip growth is modified so those branches may bulge and wrap around each other. Branching root hairs may be few in numbers, as in *Comptonia* (Callaham and Torrey 1977), or extensive, as in *Alnus* (Berry and Torrey 1983). Cell walls of deformed root hairs fluoresce yellow-orange after

staining with acridine-orange, indicating a polyanionic component not evident in normal root hairs.

Even though all root hairs may get deformed in response to the presence of *Frankia* in the rhizosphere, only one or, in some hosts, a few root hairs become infected. *Frankia* hyphae associated with deformed root hairs penetrate at the site of folding of the root hair cell wall.

At the site of wall penetration, infection hyphae are continuous with *Frankia* in the rhizosphere. Characteristic changes occur in patterns of host wall deposition, as observed in *Alnus rubra* (Inner wall layers at the site of infection resemble transfer cell walls (Berry *et al.* 1986). Within the infected hair, the microsymbiont may branch early in infection. Whether branched or unbranched, the microsymbiont become surrounded with host plasmalemma and by host derived wall material that is continuous with the wall accumulation at the site of penetration.

After *Frankia* infection, root hairs give the appearance of high metabolic activity, while uninfected hairs degenerate, infected hairs retain cytoplasmic contents, more so at the base, with numerous ribosome's and the organelles, including a nucleus, Golgi bodies, endoplasmic reticulum, and large plastids. Vacuoles are relatively small. Mitochondria are often numerous, particularly in the vicinity of *Frankia* hyphae within the cell.

2.7.4.2. Prenodule growth and infection

With the ingression of the microsymbiont, increased mitotic activity occurs in root cortex in proximity to root hair infections. Sustained cell divisions and expansions beneath the infected hair gives rise to a region called prenodule. Externally, the prenodule is recognizable, as a slight swelling of the host root, apparent before nodule lobe primordia have emerged. Encapsulated hyphae within the root hair advance into the prenodule by penetrating the cell wall at the base of the root hair and continued cell-to-cell passage. *Frankia* further proliferates in group of expanding cells in prenodule. These cells undergo continuous hypertrophy and contain dense cytoplasm with numerous small vacuoles, Golgi bodies, mitochondria, and plastids, lacking starch grains. Portions of the

cell walls of these recently expanded cells are relatively thin. *Frankia* appears to penetrate through these thin walled regions; cell-to-cell passage does not appear to take place via plasmodesmata.

2.7.4.3. Intercellular colonization

In cases reported to date, *Frankia* appears to transit from the root cortex into the developing nodule lobe primodium through cortical cells near the base of the lobe primordium. Further progress of the hyphae within the nodule lobe occurs either intercellularly or intracellularly depending on the mode of infection. In *Ceanothus*, for the most part, hyphae do not pass from cell to cell directly (Strand and Laetsch 1977), while in *Alnus*, *Casuarina*, and *Myrica*, hyphae are clearly continuous with several cells in a file or group.

Once within a nodule cortical cell, *Frankia* hyphae branch and proliferate. As the host cell grow and differentiate it deposits encapsulating wall material, as well as plasma membrane around the hyphae. *Frankia* utilizes the encapsulation material as a metabolic substrate; since it is reported to possess pectinolytic and cellulolytic activities (Safo-Sampah and Torrey 1988; Seguin and Lalonde 1989). In all of the actinorhizal genera examined to date, the *Frankia* hyphae and vesicles always remains surrounded by the host membrane and some encapsulating wall material.

The initial intercellular colonization by *Frankia* occurs in mature root cortical tissue, while at the same time nodule lobe primodium is initiated in the pericycle. Neither cell division nor hypertrophy of root cortical cells characteristic of typical prenodules is observed in *Elaeagnus*. Ultimately hyphae penetrate and invade host cells in the developing nodule lobe. In *Ceanothus* (Rhamnaceae), an intermediate situation exists in which initial invasion of the host tissue is via intercellular colonization, as in *Elaeagnus*.

2.8. Regulation of infection and nodule development

Although the processes of infection and nodule formation are complex, there are certain key molecular and cellular phenomena that are likely to be pivotal for regulation.

We presently know very little about the molecular levels of regulation. Cellular aspects of *Frankia* symbiosis that may determine developmental patterns includes the initial contact between *Frankia* and the host root cells, cell division in the host cortex, expansion of the host cell and deposition of the modified cell walls.

2.8.1. Regulation of early infections

Root exudates are one category of signaling molecule generated by the host that could regulate *Frankia* genes concerned with nodulation. Such an interaction has been demonstrated conclusively to occur in legume-Rhizobium symbiosis where specific phenolic compounds secreted in host root exudates regulate the promoters of bacterial nodulation genes. Specific phenolics, or some other regulatory signal, might also mediate compatibility between *Frankia* and actinorhizal hosts. A host exudate factor is probably involved in regulating aspects of early nodulation.

Extra cellular matrices such as mucilage, or the host derived encapsulation layer, may be important in regulating infection. Mucilage secreted by host, both in epidermal surfaces and within intercellular spaces, may facilitate bacterial colonization; *Frankia* and other microorganisms have been shown to adhere to mucilage at the root hair surface (Berry& Torrey 1983). Binding of *Frankia* to the host cell exterior could directly trigger host responses, initially at the surface and secondarily within the host cell. Additionally, the polyanionic nature of these matrices may provide a medium for flux of cations, for example, calcium, which can affect a variety of cellular phenomena.

Extra cellular polysaccharides of host origin may provide a substrate for *Frankia* enzyme activity. Seguin and lalonde (1989) observed pectinolytic activity in culture and reported positive hybridization between *Frankia* DNA and genes coding for pectate lyase. These result suggest that *Frankia* might degrade pectic substances within nodule tissues. Resulting oligosaccharides might mediate compatibility in actinorhizal associations.

2.8.2. Control of cell division centers and cell expansion

An early consequence of *Frankia* inoculation is a localized renewal of mitotic activity in cortical tissues near sites of *Frankia* ingress. Concomitantly, lateral root primordia are initiated *de novo* in the infected zone. Such stimulation of meristematic centers during early infection suggests a regulatory role for plant growth substances. Elevated levels of cytokinins, gibberellins, and auxins in root nodules have been reported providing further circumstantial evidence for the involvement of plant growth regulators in nodule development. Auxins and cytokinins have been shown to be secreted by *Frankia* (Berry *et al.*, 1989) and may also be involved in the hypertrophy of infected cells. Another possibility is that localized cell divisions or cell hypertrophy; result indirectly from a signal from *Frankia* that triggers a host derived plant growth regulator.

2.8.3. The host cell wall and *Frankia* penetration

The host cell wall is a physical barrier to microbial invasion. Penetration of host cell wall by *Frankia* may involve wall- loosening, changes in wall structure, or wall degradation. Wall degrading enzymes produced by *Frankia* could soften or dissolve host walls. *Frankia* appears to have pectate lyase gene sequences (Seguin and Lalonde, 1989). Cellulase activity has been demonstrated in *Frankia* as well (Safo-Sampah and Torrey, 1988). Endogenous pectinolytic activity is important in fruit ripening and other developmental processes and could also be involved in wall hydrolysis during nodule development. Several structural observations suggest that new wall deposition be closely linked to successful infection. *Frankia* infects host cells during cell expansion, when primary wall is deposited, both in tip growing root hairs and in promeristematic derivatives in the nodule lobe. Wall encapsulation material is deposited around the invading and proliferating hyphae. Many of the distinctive structural features of newly infected cells, including hypertrophy, the presence of numerous Golgi bodies, Golgi derived vesicles, and microtubular arrays, indicate that cell wall synthesis and deposition are primary activities at this stage.

2.9. Phylogenetic studies on actinorhizal plants and *Frankia*

Extensive studies on symbiotic nitrogen fixing associations have shown that unlike leguminous plants which form symbiotic association with rhizobia, the actinorhizal plants are considered to be polyphyletic in their origin. On the other hand, *Frankia*-the microsymbionts forming symbiotic association with these actinorhizal plants have been assumed to be phylogenetically homologous group on the basis of a variety of their morphological, biochemical, phenotypical and nitrogen fixing capacity. (Wall 2000).

A comparison of sequences and catalogs of certain regions of 16S rRNAs by Stackebrandt and his associates showed that two type-B *Frankiae* could be shown to belong to one of the major sub-lines of descent to members of actinomycete of genus *Geodermatophilus*. The 16S rRNA sequences of type-A strains have yet to be determined. Sequencing of the rRNA genes of a single *Frankia* strain showed that the 5' to 3' order is 16S-23S-5S, which is typical of bacterial in general.

A study of the phylogeny of seed plants, based on *rbcL* gene sequences, revealed that all nitrogen fixing and nodulated plants cluster in the Rosid I lineage of the angiosperms. This result suggests that the predisposition to develop nitrogen-fixing nodules of any type arose only once during the evolution of the angiosperm (Doyle 1998). Nodulated plants within the Rosid I clade can be grouped into four major lineages: three of them include actinorhizal plants (Soltis *et al.* 1995). One includes the Hamamelid families, Myricaceae, Betulaceae, and Casuarinaceae, whereas a second includes the Rosid families Elaeagnaceae, Rhamna-ceae, and Rosaceae, as well as the *Bradyrhizobium*-infected *Parasponia* (Ulmaceae). Coriariaceae and Datiscaceae define the third line of actinorhizal plants. The fourth line of nodulated plants includes the rhizobia-infected legumes of the Fabaceae. Morphologic and anatomic features of actinorhizal nodules correlate with a more detailed analysis of *rbcL* grouping (Swensen and Mullin 1997). To date, four clades of actinorhizal plants have been defined. One of the above-mentioned groups of Rosid families is divided into two sub-clades: one including Elaeagnaceae and Rhamnaceae, and the second defined by the Rosaceae (See Figure.2.2 at the end of this chapter). Fossil records and the geographical distribution of

actinorhizal species give extra support to these groupings (Benson and Clawson 2000). Phylogenetic studies on *Frankia* have focused mainly on 16S RNA gene sequences (Benson *et al.* 1996; Jeong *et al.* 1999; Normand *et al.* 1996; Ritchie and Myrold 1999). Similar results have been obtained with *nifD* gene sequences (Normand et al.1992), and recently confirmed using *recA* and *glnII* sequences (Cournoyer and Lavire 1999). In all these studies, there has been difficulty with isolating *Frankia* from root nodules. This problem has been partially overcome by direct amplification of total nodule DNA, using specifically designed primers for *Frankia.* Consensus phylogenetic trees generated from 16S rDNA sequences consistently yield three major groups of *Frankia,* and a fourth "*Frankia*–like" clade of Nod. /Fix. actinomycetes (See Figure2.2 at the end of this chapter). Subgroups can be found, although these are not statistically well supported. There are many well-known isolates included in groups I and II, whereas no one isolate has been obtained from group III, which is defined only on the basis of analysis of nodule-extracted DNA. Physiologically, at least, the absence of a septum in vesicles of nodules of host plants infected with group III *Frankia* agrees with the proposed division. Although the phylogenies of the microsymbiont are not congruent with the four host clades, a close relationship exists between the plant and bacterial groups. Further analysis shows that the plant clades diverged earlier than the *Frankia* clades, suggesting that the *Frankia*-actinorhizal symbiosis evolved independently, at least three or four times, rather than co-evolving from an ancestral symbioses (Benson and Clawson 2000; Jeong *et al.* 1999; Swensen 1996). Nevertheless, once the symbiosis was established, the plants or *Frankia* were retained within certain taxonomic groups, with limited lateral transfer and probable co-evolution from that point onwards (Simonet *et al.* 1998). These analyses as a whole reinforce a model for host preferences of specificity in actinorhizal symbioses. (Wall 2000).

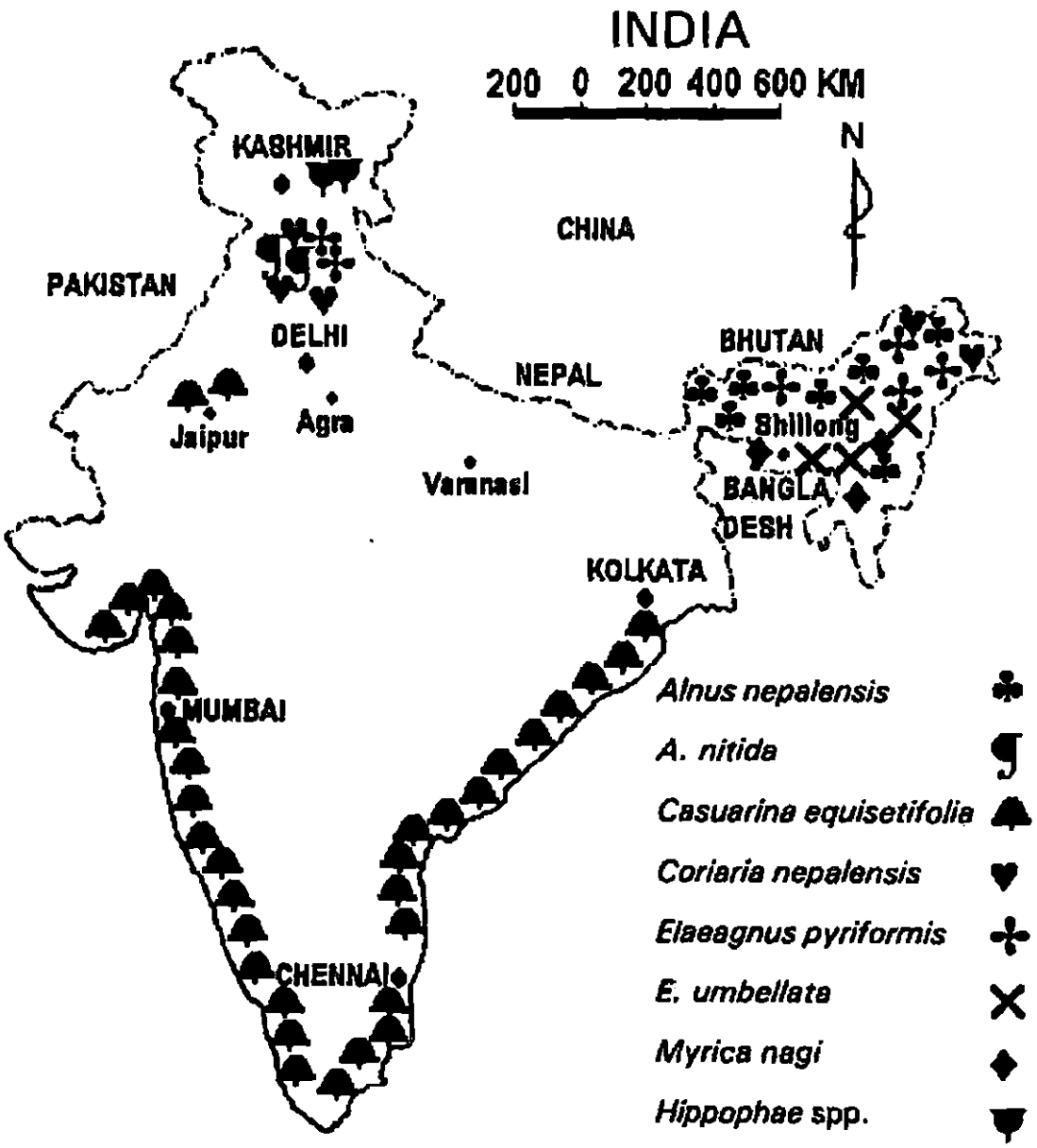

Figure 2.1: Distribution of actinorhizal plants in India

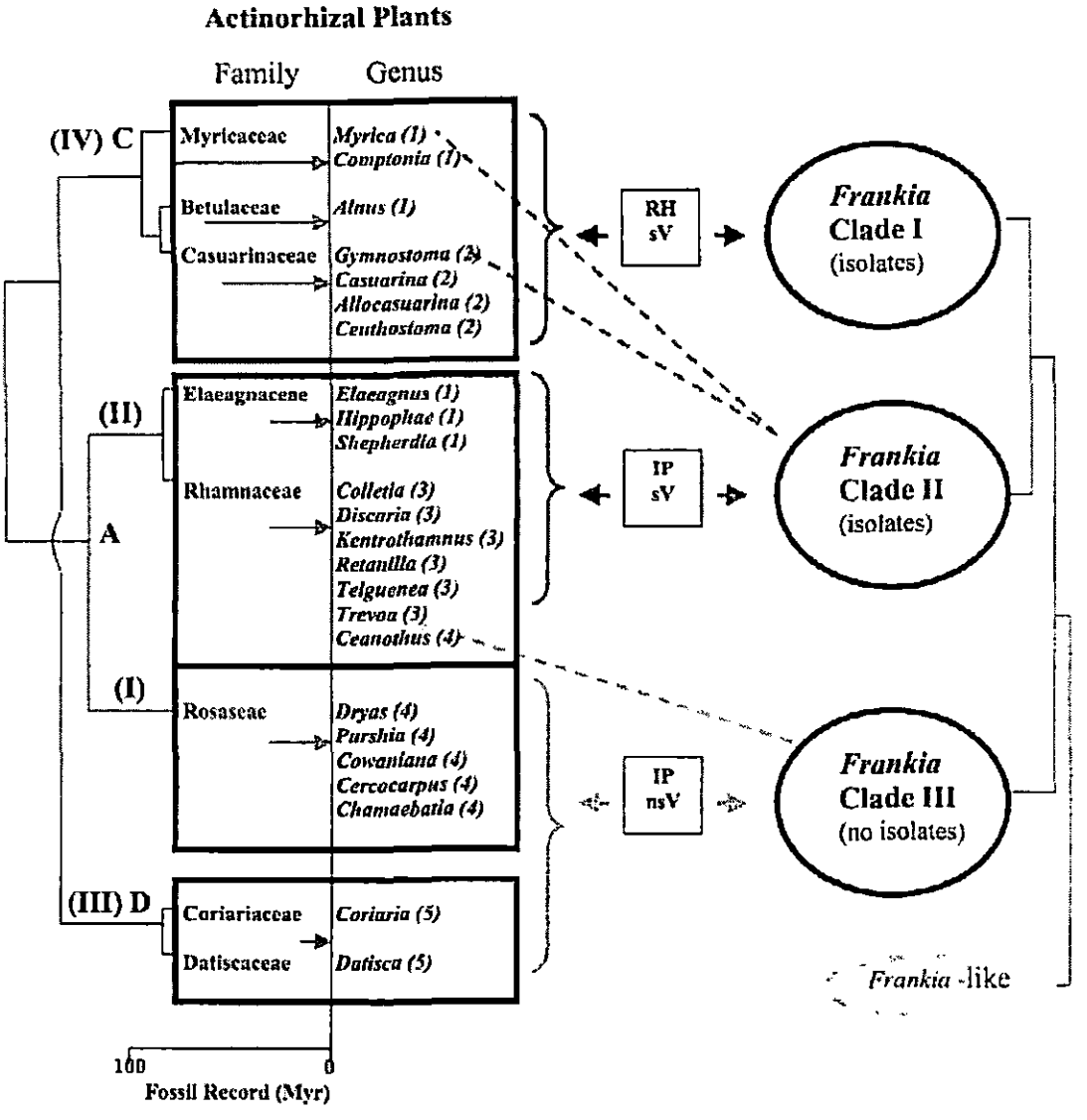

FIGURE 2.2: Phylogenetic grouping of actinorhizal plants and *Frankia*. Number between brackets of plant genus indicates native geographical distribution *(1)* to most continents, *(2)* to Australia and western Pacific, *(3)* to South America and southern New Zealand, *(4)* western North America, *(5)* disjunct distribution in northern and southern temperate zones. *RH,* root hair infection; *IP,* intercellular penetration; *sV,* septated vesicles in nodule; *ns* V, nonseptated vesicles in nodule. Based on Benson and Clawson (2000); Jeong *et al.*, (1999); Huss-Danell (1997). Groups I–IV proposed by Soltis *et al.* (1995); Clades A–D proposed by Swensen and Mullin (1977). Adapted from L.G.Wall (2000).

TABLE: 2.1 Two types of Frankial strains on the basis of physiological difference:

Type A	**Type B**
They are physiologically active.	They are physiologically inactive.
They grow relatively rapidly.	They grow very slowly.
They are aerobic and can be maintained on slants.	They are strictly microaerophilic and cannot be maintained on slants.
They do not reinfect host plant from which they are isolated, at least in the experimental conditions.	They can infect the host plant from which they are taken.
They utilize a variety of monosaccharides and disaccharides with or without acid production.	They cannot utilize carbohydrates, proteins, or starch. The preferred carbon sources are organic acids or between compounds.
Many of them produce hydrolytic enzymes, such as pectinases, cellulases, amylases, and proteases.	No such reports.

Compiled from Lechevalier (1994).

TABLE 2.2: Frankial isolates in symbiotic association with Actinorhizal plant genera

ORDER	FAMILY	GENUS	NUMBER OF SPECIES	ISOLATES
Casuarinales	Casuarinaceae	*Allocasuarina*	54	+,I,E
		Casuarina	16	+,I,E
		Ceuthostoma	2	-
		Gymnostoma	18	+,I,E
Fagales	Betulaceae	*Alnus*	47	+,I,E
Myricales	Myricaceae	*Comptonia*	1	+,I,E
		Myrica	28	+,I,E
Protales	Elaeagnaceae	*Elaeagnus*	38	+,I,E
		Hippophae	2	+,I,E
		Shepherdia	2	+,I,E
Ranunculales	Coriariaceae	*Coriaria*	16	+,N
Rhamnales	Rhamnaceae	*Ceanothus*	31	+,N
		Colletia	4	+,N
		Discaria	5	+,N
		Kentrothamnus	1	-
		Retanilla	2	+,N
		Taalguenea	1	+,?
		Trevoa	2	+,N
Rosales	Rosaceae	*Cercocarpus*	4	+,N
		Chamaebatia	1	-
		Cowania	1	+,?
		Drayas	3	-
		Purshia	2	+,N
Violales	Datiscaceae	*Datisca*	2	+,N

Symbols: (-)Isolates not reported; (+)Isolates obtained; (I)Infective; (N)Non-infective; (E)Effective in fixing Nitrogen; (?)Ineffectiveness unknown or unreported. Compiled from: Bond (1983), Torrey & Berg (1988), Newcomb & Wood (1987), Baker & Schwintzer (1990) and Benson & Silvester (1993).

TABLE 2.3: Continent wise distribution of native actinorhizal families and genera.

Continent	Family	*Genus*
North America	Betulaceae	*Alnus*
	Coriariaceae	*Coriaria*
	Datiscaceae	*Datisca*
	Elaeagnaceae	*Elaeagnus*
		Shepherdia
	Myricaceae	*Myrica*
		Comptonia
	Rhamnaceae	*Ceanothus*
	Rosaceae	*Cercocarpus*
		Chamaebatia
		Cowania
		Dryas
		Purshia
South America	Betulaceae	*Alnus*
	Coriariaceae	*Coriaria*
	Myricaceae	*Myrica*
	Rhamnaceae	*Adolphia*
		Colletia
		Discaria
		Kentrothamnus
		Retanilla
		Talguenea
		Trevoa
Africa	Myricaceae	*Myrica*
Eurasia	Betulaceae	*Alnus*
	Coriariaceae	*Coriaria*
	Datiscaceae	*Datisca*
	Elaeagnaceae	*Elaeagnus*
		Hippophae
	Myricaceae	*Myrica*
	Rosaceae	*Dryas*
Australia and Oceania	Casuarinaceae	*Allocasuarina*
		Casuarina
		Ceuthostoma
		Gymnostoma
	Coriariaceae	*Coriaria*
	Myricaceae	*Myrica*
	Rhamnaceae	*Discaria*

Adapted from Baker (1988) and Baker and Schwintzer (1990) and update based on Cruz-Cisneros and Valdes (1991).

CHAPTER 3

Materials and Methods

3.1. Isolation and Identification of *Frankia*

3.1.1. Germplasm collection

Germplasm was collected from different sites of Darjeeling (West Bengal), India in the month of February and November. The nodules were collected from *Alnus nepalensis* trees found in various regions of Darjeeling. The samples were traced on the way from Siliguri to Darjeeling via Mirik namely Thurbo, Fatak, Pashupati, Ghoom, and Darjeeling (See Figure.3.1 at the end of this chapter). In the field, the plant species were first located, essential data like its general information, habit, habitat, area of vegetation, specific collection site, soil type and nodules were recorded (See Figure.3.2 at the end of this chapter). Some healthy and young trees were chosen for nodule collection. Photographs of *Alnus nepalensis* and the surrounding vegetation were taken (PlateIII). The vegetation around the tree of our interest was studied and recorded as follows (See table 3.1 at the end of this chapter)

During collection of samples from all the sites one thing was strictly followed i.e. only the nodules of light brown colour were collected since the light colour of nodule is an indication of its active growth and youngness (Myrold 1994).

The percentage of nodulation was calculated by the following formula (Raman & Elumalai 1991.)

$$\% \text{ of nodulation} = \frac{\text{Number of plants with nodules}}{\text{Number of plants observed}} \times 100$$

In both the cases collected nodules were stored in plastic bags containing moistened paper towel to maintain favorable water potential and kept in an icebox containing ice to limit tissue deterioration and slow down the growth of contaminating microorganisms throughout the journey. Soil samples were also collected in much the same manner. Samples of nodules and soil were stored at -20°C immediately after bringing to the laboratory.

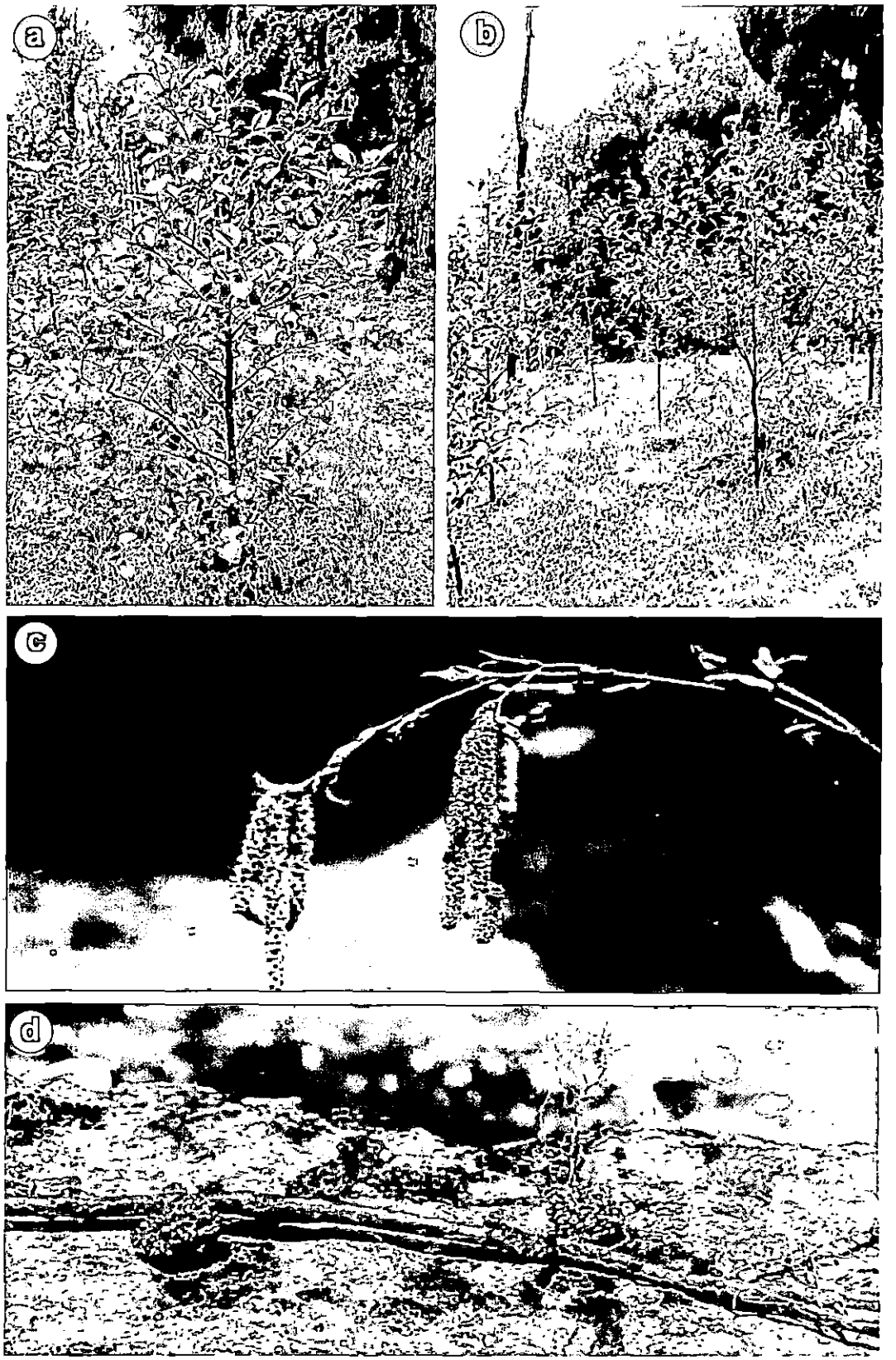

Plate III

(a&b) *Alnus nepalensis.* Two collection sites of *Frankia* germplasm of Darjeeling. (c) An inflorescence of *Alnus nepalensis* (d) A root nodule clump from *Alnus nepalensis.*

3.1.2. Isolation of endophyte-the microsymbiont

3.1.2.1. Nodule sterilization

Nodules were cleaned in plain water to remove soil and organic debris. Nodule lobes were separated and further cleaned by washing several times in a mild detergent such as Extran (E Merck, India cat#MA02). Lastly they were washed several times with distilled water and then with sterilized distilled water. Since *Frankia* is a slow growing organism, a chance of contamination of its culture by soil borne fast growing microorganisms is more (Lechevalier 1994). To overcome this problem surface sterilization of nodules were done by following methods:

- Nodules were treated with 0.1% $HgCl_2$ (E Merck India Cat# 17524) for 2 minutes and washed 6-7 times with sterile double distilled water.
- Nodules were treated with 30% H_2O_2 (E Merck India Cat# 1.07209.0250) for 5 minutes and then washed several times in Petri plates with sterile double distilled water.

3.1.2.2. Isolation of *Frankia* in liquid culture

After proper sterilization of the nodules following isolation techniques have been tried.

- A single surface sterilized nodule lobe was taken on a sterilized slide.
- The upper epidermal part was peeled off using two sterilized needles.
- The nodule lobe was then washed twice with sterile distilled water.
- The peeled nodule lobes were crushed on the side of the culture tubes containing liquid growth medium for *Frankia*.
- Surface sterilized nodules were chopped into small pieces and put into liquid medium. The tubes were incubated at 28-30°

3.1.2.3. Common media for isolation

Although there exists a range of isolation medium for *Frankia* e.g. DPM used by (Baker and O Keefe 1984), F medium used by (Simonet et al. 1985), OS –1 by (Dobritsa and Stupar 1989) and Q- mod by (Lalonde & Calvert 1979) but the best medium like

DPM by (Baker and O Keefe 1984), was finally used after few trial and error methods (See appendix I for composition of media).

3.2. Plant infectivity tests

3.2.1. Germination of seed and seedling cultivation

Surface sterilization of seeds was done with 30% H_2O_2 for 10 *minutes* and it was rinsed several times with sterile distilled water. The seeds of *Alnus* were placed on a sterile moist filter paper and kept in the BOD incubator at 26 ± 3^{0}C for germination.
Fifteen days old seedlings were transferred to sterile pouches containing different concentrations of Hoagland solutions (1/4, 1/8, 1/16) without nitrogen (See appendix I for composition). The following three sets were prepared for each isolate:

- Seedling inoculated with crushed nodule suspension (+ve control) (Section 3.1.2.3. for preparation of nodule suspension).
- Seedlings inoculated with 100µl of 30 days old *Frankia* culture under test.
- Un-inoculated seedlings were used in each set as –ve control.
- Each set contained twenty seedlings. The *Alnus* seedlings were allowed to grow in a plant growth chamber at 26°C with approximately 90% humidity and 1100 lux illumination.

3.3. Nitrogenase activity of *Frankia* in pure culture

Four cultures two from Thurbo (AnTB1& AnTB2) and two from Ghoom (AnGM2& AnGM5) were tested for nitrogenase activity.
Acetylene reduction assay (ARA) (Burris, 1967) was employed to measure nitrogenase activity of the isolates. For measuring the nitrogenase activity 3 ml of 30 day old subculture of each isolate was taken in 9 ml stoppered vials, 0.9ml of air was replaced with equal volume of acetylene. The vials were incubated at 28±1°C and were shaken time to time. Ethylene produced from acetylene was measured with a "Perkin Elmer-8700" gas chromatograph fitted with Porapak–T column. The chromatograph used hydrogen as a fuel and nitrogen serves as carrier gas.

3.4 Post isolation workup

3.4.1. Incubation and sub culturing

The vials thus inoculated were protected by properly sealing them with Parafim M (American National Can™ Chicago.60631) and were incubated in the dark at 28°C for several weeks to one month. The cultures obtained were initially studied under phase contrast microscope for the characteristic sub-surface sporangia or presence of vesicles. Selected colonies were aseptically withdrawn from liquid culture and subcultured in fresh medium. Colonies selected for sub culturing need to be thoroughly homogenized. This can be done by grinding the colony with sterile buffer or medium in small tissue grinder. Homogenization increases the number of actively growing hyphal tips and also helps to release spores from sporangia. The homogenized suspension is then inoculated into the fresh medium.

3.4.2. Maintenance of culture

After successful isolation of *Frankia* in pure culture, the isolates were named as per the conventional nomenclature systems of Frankia, i.e. first two letters from the Genus and species of the host plant with the first one Capitalized and the subsequent two letters from the collection site. For instance, culture from the nodule collected from Thurbo was named as AnTB1, AnTB2 etc. The cultures were maintained in DPM medium. To maintain active culture, sub-culturing was done every 2 to 6 months. Cultures were homogenized while transferring them to break apart hyphae and promote more rapid growth.

3.4.3. Decontamination

Because of slow growing nature, *Frankia* cultures sometimes become contaminated. Decontamination was achieved in several ways. Different antimicrobial agents were used like 500µg/ml of cyclohexamide (Actidione, Sigma, USA. Cat#C-2609) for removing of fungal contaminants. Actidione inhibits protein biosynthesis in eukaryotic cells by binding 80S ribosome. Since *Frankia* is gram +ve bacteria, Nalidixic acid (10µg/ml) (1-ethyl-4-dihydro-7methyl-4-oxo-1, 8-napthyridine-3-carboxylic acid,

(Sigma, USA. Cat#N-3143) was used. The efficiency of either decontamination approach is enhanced by re-culturing on an N-free medium (DPM) and further streaking this enrichment on N-free agar to re-isolate *Frankia*.

3.5. Structure and ultra structure

3.5.1. Light Microscopy

Root nodules, were fixed in FAA (90ml of 70% ethanol, 5ml glacial acetic acid and 5ml Formalene). They were then dehydrated in a series of extra pure acetone (E Merck India) and embedded in paraffin wax. Transverse and longitudinal sections 6-10μm thick were cut with a microtome (Leitz 1512, Germany). The safranin- fast green staining procedure (Sass 1958) was used to stain the cortical and xylem cells differentially in order to study their relative arrangement. The *Frankia!* culture was stained according to the procedure of Gram's staining protocol (Cappuccino/Sherman 1996).

3.5.2 Scanning Electron Microscopy (SEM) of *Frankia in vivo & in vitro*

The SEM provides information on specimen surface (fine surface topography). It offers a three-dimensional surface view of solid tissue. Both pure cultures as well as *Frankia* within nodules were taken for SEM. General requirements of SEM of *Frankia* (both *in-vivo* & *in-vitro*) are as follows:

3.5.2.1 Chemical fixation

Fixation offers the best way to preserve the cells as close as possible to their in-vivo condition with respect to volume, morphology and spatial relationships of organelles and macromolecules.

3.5.2.1.1 For *Frankia* in pure culture

One-month-old *Frankia* culture was taken in a micro centrifuge tube and allowed to settle down. The upper aqueous phase containing the medium was decanted carefully. The cells were fixed by adding 1ml of 3% glutraldehyde and kept for 4 hours.

Glutraldehyde was then decanted carefully and the cells were stuck on glass stubs with the help of egg albumin. The cells were then washed thoroughly in 0.1 M cacodylate buffer (see appendix II for composition) and post fixation was done in 1% OsO4 for 2 hours. It is conclusive that although the osmolality, pH and total time allowed for fixation are all critical in obtaining optimal preservation, there is a range of satisfactory conditions for particular specimen.

3.5.2.1.2. For *Frankia* inside nodules

Nodule lobes were taken, each nodule separated and washed thoroughly with distilled water. The nodule lobes were then fixed with 3% glutraldehyde for 4 hours and then washed with 0.1M cacodylate buffer. They were then post fixed in 1% OsO_4 for 2 hours.

3.5.2.2. Dehydration

A dry specimen is required by the design of scanning electron microscope. Drying in air causes gross distortion of cells due to the action of surface tension. So, to achieve distortion free drying, critical point drying is necessary. Since water is immiscible with most of currently used transitional fluids necessary to the critical point method, removal of water is done by the use of dehydration fluids; most commonly used among them is acetone or alcohol in ascending concentrations. Therefore in both cases dehydration was done with a series of acetone (30%, 50%, 70%, 80%, 90%, 95%), keeping then in each grade for 30 minutes with two changes followed by one hour in dry acetone with two changes. However, nodules were kept for 24 hours in each grade for proper dehydration. After dehydration, nodules were embedded in paraffin wax and thin sections (10μm thick) were prepared with the help of microtome (Leitz 1512, Germany). The sections were then mounted on glass stubs and made wax free with the help of xylene.

3.5.2.3. Critical point drying

Critical point freeze drying of the dehydrated *Frankia* cells was done using a Polaron E 3000 apparatus. The drying was carried out at 31.5°C at 1100 PSV. Critical

point drying which achieves transition from liquid to the vapor phase without the passage of a phase boundary and the associated deforming forces due to surface tension is the most popular method of drying specimens for SEM. After drying the cells were mounted for coating.

3.5.2.4. Coating

Coating means deposition of a heavy metal onto the surface of the specimen to make it electrically and thermally conductive. Conductivity is necessary for two reasons:

- To avoid image artifacts commonly referred to as charging and
- To acheive, a high yield of secondary electrons upon electron bombardment of the specimen and good resolution in the scanning electron microscope.
- Metal coatings can be established by evaporation or sputtering. For *Frankia* cells, it was done by gold sputtering.

3.5.2.5. Gold sputtering

Gold coating of *Frankia* cells and nodule sections was done with JEOL Fine Coat JFC-1100 ion sputter under vacuum in an inert atmosphere. Coating of uniform thickness was obtained by evaporating gold on the cells kept at specific distance at specific time. The specimen was then fixed on metal tablets with Dolite electroconductives and observed under scanning electron microscope (JEOL JSM – 35CF).

3.6. Isolation of genomic DNA

3.6.1. Isolation of genomic DNA from pure culture

Following protocol was followed for genomic DNA isolation of *Frankia* from pure culture:

- One ml of 1 month old *Frankia* culture was taken in a micro centrifuge tube
- It was centrifuged at 8500Xg for 10 minutes and the supernatant was discarded.
- The pellet was resuspended in 1ml of TE (pH 8.0) buffer (See appendix II for composition) and transferred to a 1.5ml micro centrifuge tube (Tarsons, India).

- It was centrifuged at 8500Xg for 15 minutes and the pellet was dissolved in 1ml of TE (pH 8.0) buffer.
- 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 chemical Co., USA. Cat#L-6876) and a pinch of achromopeptidase (Sigma Chemical Co., USA. Cat#A3422) were added and was incubated at 20°C for one hour.
- 250µl of 20% SDS was added. It was incubated at 60°C for 30 minutes and at room temperature for 15 minutes.
- The solution was then divided into two equal parts, 625µl/tube.
- 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 8500Xg for 10 minutes and the upper aqueous phase so obtained was taken in a fresh tube.
- An equal volume of Chloroform (600µl) (E Merck Ind. Ltd. Cat#822265) was added gently and centrifuged at 8500Xg for 5minutes.
- The upper aqueous phase was taken in a fresh tube and to it 360µl of Isopropyl alcohol (E Merck Ind. Ltd. Cat#17813) was added and the tube was kept at room temperature overnight.
- The tubes were then centrifuged at 4°C for 20minutes at 12000Xg.The supernatant was discarded.
- The pellet was washed with 70% alcohol.
- Finally the pellet was dried in vacuum desiccators and the DNA was resuspended in 10µl of Pyrogen free water.

3.6.2. Isolation of genomic DNA from Alder root nodules

Ten different *Alnus nepalensis* trees were chosen from different collection sites; one from Pashupati, three from Fatak, three from Thurbo, two from Ghoom and one from New Fatak. From each tree ten nodules were collected. The nodules were numbered as

AnP from Pashupati, AnG from Ghoom, AnT from Thurbo, AnF from Fatak and AnNF from New Fatak. These samples were taken for RFLP based diversity studies. The surrounding vegetation of desired tree was cleared off and the digging of the soil was initiated at the base of the stem and worked towards the root tip. The root nodules were found at a reachable depth of 3"-12" and carefully excavated along with a small portion of the attached root system.

Various factors like size of the nodules and the presence of plant phenolics make the isolation of DNA from alder root nodules a tedious process. We adopted the method of Rouvier *et al.* (1996) with few modifications as mentioned below:

- A few nodule lobes were first washed with distilled water and then surface sterilized with 30% Hydrogen Peroxide for 1 minute. They were then washed in autoclaved distilled water twice.
- The epidermal part of a single nodule lobe taken was peeled in PVPP peeling buffer (See appendix II for composition) with the help of two fine needles.
- Each of these peeled nodule lobe was then crushed in a 1.5ml centrifuge tube containing 300µl of pre-warmed DNA-Extraction buffer (See appendix II for composition).
- After complete crushing, the final volume of the extraction buffer was made up to 500µl and it was then incubated at 65°C for 1 hour. This solution was then centrifuged for 10 minutes at 10000Xg at 4°C.
- The supernatant was carefully pippetted out & transferred to a fresh 1.5ml tube and extracted twice with equal volume of chloroform:isoamyl alcohol (24:1). After centrifugation at 12000Xg for 10 minutes, the aqueous phase was taken in a fresh 1.5ml tube.
- The supernatant containing DNA was then precipitated with double volume of absolute alcohol (E Merck Germany Cat#K29824783). The solution was then centrifuged at 12000Xg for 20 minutes at 4°C. The pellet was washed with ice cold 70% EtOH and dried in vacuum desiccators.
- DNA pellet was then dissolved in 20µl of pyrogen free water. This method yields DNA preparation, which was clear in appearance.

Further modifying Rouvier *et al* (1996) method, we did RNase treatment of the isolated DNA to digest the RNA present in the DNA.

3.7. RNase treatment:

To this DNA, RNase (Sigma Cat# R-4875) was added at a rate of 50μgml^{-1} and incubated for 1 hour at 37°C in a cooling dry bath (Genei, India).

- To remove RNase the DNA was extracted with an equal volume of PCI (25:24:1) and centrifuged at 8500Xg for 5 minutes at room temperature.
- The upper aqueous phase was re-extracted with CI (24:1) and again centrifuged at 8500Xg for 5 minutes at room temperature.
- The supernatant so obtained was precipitated with double volume absolute alcohol and the pellet obtained was washed with 70% alcohol, vaccum dried and dissolved in 20μl Pyrogen free water.

3.8. Agarose Gel Electrophoresis of Genomic DNA both from pure culture and root nodules.

The size of the DNA was analyzed by using the following protocol:

- Agarose gel was prepared using Molecular biology grade, DNAase free 0.8% agarose (gelling temperature 36°C) (Sigma Cat#A 9539). O.24gm of agarose was dissolved in 30ml of 1X TBE buffer. (See appendix II for composition).
- This was heated in a microwave oven. (LG make).
- After cooling it to a temperature of 40°C, 0.5μg/ml Ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenaanthridinium bromide, $C_{12}H_{20}BrN_3$, M_r 394.33, (Sigma Cat#E 8751) was added to the gel for staining DNA.
- The gel was cast on a gel platform (100×70mm) (Tarsons Cat#7024). An eight well comb was inserted to make wells.
- After solidification, the gel platform along with the solid gel was put into a Mini Submarine Gel Electrophoresis Unit (Tarsons Cat#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).

- 2µl of DNA was mixed with 3µl loading buffer (type III, Sambrook *et al.* 2001) (for composition and preparation see appendix II) and was loaded into the wells.
- Lambda DNA *Hind III* was used as a molecular marker (Bangalore Genei Cat# MBD 2L).
- The DNA was made to run at a constant volt of 35V applied with Electrophoresis Power Supply Unit (Tarsons Cat#7090).
- After 1 hour the gel was viewed on a UV Transilluminator. (Bangalore Genei Cat#SF850).

3.9. DNA estimation

The yield and the purity of DNA were checked with the help of Spectrophotometer (Thermospectronic UV1) from the absorbance data of DNA samples at 260nm and 280nm. 2µl of DNA was diluted to 2ml with the help of pyrogen free water. The solution was then measured in a spectrophotometer at both 260nm and 280nm. Purity of DNA sample was calculated from ratio of O.D 260/O.D 280 and the quantity of DNA was estimated assuming 1 $O.D_{260}$=50µg.ml^{-1}.

3.10. PCR Amplification of genomic DNA both from the nodules and cultures

3.10.1. DNA Amplification

Double stranded DNA amplification was performed and the proximal, middle, distal and *nif* IGS-HD of the total genomic DNA of *Frankia* were amplified with the help of *Frankia* specific primers synthesized from Sigma-Genosys. The list of the primers is given in a tabular form (See table 3.2 at the end of this chapter).

The primers were dissolved in 1X TE (See appendix II for composition) buffer for making 100µM mother stock and were used at a final concentration of 0.5µM.

Different concentrations of template DNA like 10-50ng was used to amplify different gene regions (See table 3.3 at the end of this chapter).

The reaction was performed with the GeneAmp 2400 thermal cycler (Perkin Elmer, USA) under the following conditions: initial denaturation for 5 min. at 94°C, 35 cycles of denaturation (1 min. at 94°C), annealing (1 min.) and extension (1 min. at 72°C), and a final extension at 72°C. The reaction volume was 50µl.

Annealing temperature depends upon the size and nature of the primers and was calculated with the following formula.

Annealing temp. (°C)= {2(A+T)+4(G+C)}-5

Though both the primers (Forward and Reverse) may be having different annealing temperature, in such case the lowest one was used.

3.10.2. Gel electrophoresis

5µl of the amplified product was analyzed by performing the gel electrophoresis as mentioned above.

3.10.3. Gel documentation

Photographs of the amplified PCR products were taken with Kodak digital Science DC-120 Digital Camera.

3.11. Purification of amplified PCR products

Purification of the amplified PCR products was done using the Sigma GenElute™ PCR Clean-Up Kit as per the manufacturer's protocol. This purified amplification product was further used for cloning and RFLP studies.

3.12. Cloning of PCR products

A part of PCR product has been cloned into T-Vector with the help of TA Cloning kit supplied by Bangalore Genei, India.

3.12.1. Properties of T vector:

- Lac promoter: Expression of lac Z (α fragment) that helps in α-complementation. (Blue/White screening).

- LacZ α-fragment: Encodes for the first 146 aminoacids of β-galactosidase.
- Ampicillin resistance gene: Selection and maintenance in E.Coli.
- pMBI: The origin of replication, maintenance and high copy number in E. coli.
- T-7 Promoter: *in vivo/in vitro* transcription of RNA.
- SP6 Promoter: Sequencing of insert.
- f1 origin: Rescue of strand for mutagenesis and single strand sequencing.
- Multiple cloning sites: selection of restriction sites for cloning.

  (See figure 3.3 at the end of this chapter)

3.12.2. Ligation into T-Vector:

Two µl (50ng) of T-Vector was used for each reaction. Amount of PCR product to be used was calculated by following formula.

$$\frac{\text{Size of PCR product (bp)} \times \text{50ng vector}}{\text{3000bp (size of the vector)}}$$

The concentration of PCR product has been determined by spectrophotometer as mentioned before.

3.12.3. Ligation of reaction:

Set up of ligation reaction was as follows.

- PCR product-Xµl
- 10X ligation buffer-1µl
- T-Vector-2 (50ng) µl
- T4 DNA ligase-1µl

The volume was made upto 10 µl with distilled deionized P.F.water. The ligation reaction was incubated at 16°C in a ligation bath (Bangalore Genei Cat # LIB-1) for 4-6 hours followed by freezing the ligation mixture in 20°C until further use.

3.12.4. Preparation of Bacterial Transformation

3.12.4.1. Preparation of competent cells:

- An overnight grown culture of DH5∝ was taken in a prechilled centrifuge tube and centrifuged at 5000-7000rpm for 15minutes at 4°C.

- The supernatant was discarded and the pellet was suspended in 100mM $CaCl_2$ solution.
- It was then kept on ice for 30 minutes and the cells were spinned down at 500rpm for5 minute at 4°C.
- The pellet was resuspended in 5ml 100mM $CaCl_2$ and kept on ice for 30 minutes and was used for transformation.

3.12.4.2. Bacterial Transformation:

- The water bath was kept ready at 42°C.
- The solid L.B. medium plates (See appendix I for composition) containing antibiotic, X-gal & IPTG were prepared. X-gal and IPTG were added only at the time of plating the transformed cells.
- 100 µl of competent cells were at first thawed on ice and 10 µl of ligation mix was added directly into it and then mixed gently.
- Vials were incubated on ice for 30 minutes.
- Heat shock at 42°C was given to the mixture and then placed it on ice immediately.
- To each of these vials 400 µl of L.B. medium (For composition see appendixI) was added.
- The vials were incubated on 37°C shaker at 225 rpm for 1 hour.
- 250 µl of it was spread on to the antibiotic, Xgal and IPTG containing L.B. plates.
- The plates were then incubated overnight at 37°C. For colour development

3.12.5. Screening and analysis:

From the plates that developed blue and white colonies, 10-20 white colonies were taken into 5ml L.B. medium containing 100µg/ml of ampicillin and grown overnight.

Plasmid isolation was performed with the help of Silica column based plasmid mini prep. kit (GenElute™ Sigma Aldrich USA Cat# PLN70) as per the manufacturer's protocol.

The distal part of 16S rRNA gene fragment was digested out with *Nco* I (See Table 3.4 at the end of this chapter) since the insert was flanked by two *NcoI* sites and the fragment (Distal part of 16S rRNA gene does not have any *NcoI* sites.

Region of *nif* H-D IGS however had *NcoI* sites. Therefore this fragment was digested out with the help of two restriction enzymes, *PstI* and *ApaI*. Reaction conditions and the assay buffer for these two enzymes were different (See Table 3.4 at the end of this chapter). Therefore we first cut the plasmid with *PstI*, the product was purified with Sigma Spin™ post reaction clean up column and then cut with *ApaI*.

3.13. Restriction digestion of amplified regions.

3.13.1. Preparation of Restriction digestion reaction mix:

- 10µl of PCR amplified product of *nif* IGS-HD and other regions as described in section H.1 was taken in a sterile microcentrifuge tube.
- 2µl of 10X restriction enzyme digestion buffer (supplied with Bangalore Genei Pvt Ltd. restriction enzymes), was added to it and mixed thoroughly.
- 5U of restriction enzymes (Bangalore Genei Pvt. Ltd.) was used for restriction digestion. The enzymes used are shown in Table 3.5 (See table 3.5 at the end of this chapter).
- The tubes were incubated in a cooling dry bath at indicated reaction temperatures for 1 hour.
- The reaction was stopped by adding 0.5M EDTA (pH 8.0) to a final concentration of 10mM. The digested DNA was stored at -20°C until further use.

3.13.2. Analysis of restriction fragments (RFLP)

RFLP analysis was done performing the gel electrophoresis of the digested product.

A 3% (w/v) molecular biology grade high resolution agarose (Sigma, USA) gel was prepared in 1X TBE for a 15X10 cm casting tray. Electrophoresis was done in midi submarine electrophoresis unit (Tarsons Cat#7050). Twenty µl of the digested DNA was electrophoresed in 3V/cm electric field.

Lambda DNA *Hind* III digest Bangalore Genei, Cat#MBD2S) and Gene Ruler™ 1Kb ladder (MBI Fermentas, Lithuania Cat#SM0311) were used as molecular wt. standards. The gel was visualized on a UV transilluminator (Gibco-BRL) and photographed using Kodak digital Science DC-120 Digital Camera.

3.13.3. Scoring of data

The PCR RFLP data were scored as present (1) or absent (0) using the SIMQUAL program and DICE coefficients. A matrix was computed and phonograms developed by a UPGMA clustering of the matrix NTSYS-pc (Rohlf 1994).

3.14. The influence of host on nitrogenase activity

In many ecosystems plant growth is limited by the availability of nitrogen suggesting that dinitrogen fixing plants should have a substantial advantage. In fact nitrogen fixing symbioses involving both actinorhizal plant and *Frankia* have evolved and play a permanent role in the wide variety of ecosystem. Though it is the Frankia which actually fix the atmospheric Nitrogen, earlier work revealed that the host may play a greater role in developing the symbiosis (Sougofara *et al.* 1992). In our earlier experiments we found that amount of Nitrogen fixation may vary significantly from one plant to its neighbouring one.

This may be due to several factors like the age of the host plant, pH of the soil, the age of the nodules, genetic variation among the host and the symbiont etc. In our present study we have chosen an Alder stand in Tadong area of Gangtok (Sikkim). These trees are young and are of same age since they are all planted at the same time. Fresh light brownish nodules were collected in the month of April from 10 different trees of same Alder stand (Section 3.1.1). From the site seventeen healthy trees were dug for the nodule collection and the soil samples were collected for pH evaluation. In around 60% of the trees nodules were found. The plants were chosen carefully so that they are equally healthy and were of same height. Along with the nodules the temperature was recorded. Ten nodules from each plant were used for ARA. All the other general information about the site of collection was also collected as mentioned in 3.1.1 section. The selected site

vegetation as studied also included few more plant species other than *Alnus nepalensis* e.g. among the tree species were *Erythrina arborea, Exbucklandia populnea, Elaeocarpus sikkimensis* etc. and among shrubs most common were *Cesternum auranticum, Osbeckia chinensis and Melastoma paniculata* etc as identified and recorded at the site of collection.

For ARA each nodule was surface sterilized carefully and weighed. A vial was taken and its volume was measured (7.672ml). The vial was then fitted with a tight serum stopper. A small moist strip of paper 2cm/2cm were placed in the vial and the nodules were placed on top of it. The vial was sealed and C_2H_2 was injected. The amount of C_2H_2 was 0.76ml (10% of the volume of air was removed through the stopper prior to the addition of C_2H_2 and incubated at ambient conditions for 1-2 h. Sampling of C_2H_2 was done using a gas tight hypodermic syringe and injected into the "Perkin Elmer-8700"gas chromatograph. The amount of acetylene reduced to ethylene was calculated and expressed as nano mol/g/hr (Burris 1967). Each of the nodules which were used in ARA experiment were subjected to total DNA amplification as per the previous protocol discussed. DNA of the samples was amplified with different primers (See Table 3.2 at the end of this chapter). The amplified products were subjected to restriction digestion using the enzymes as mentioned in (See Table3.5 at the end of this chapter). These data were scored as (1) and (0) further analyzed with the help of a UPGMA software NTSYSpc (Rohlf 1994).

Table 3.1 Associated spp. of *Alnus nepalensis*

FAMILY	GENUS	SPECIES
Asteraceae	*Anaphelles*	*contorta*
Cyperidaceae	*Cyperus*	sp.
Asteraceae	*Eregeron*	*Karwinskianus*
Asteraceae	*Eupatorium*	*glandulosum*
Apiaceae	*Hydrocotyl*	*himalaica*
Polygonaceae	*Persicaria*	*nepalensis*
Urticaceae	*Pauzolzia*	*hierta*
Rubiaceae	*Rubia*	*manzit*
Caprifoliaceae	*Vivernum*	*eruvescens*

Table3.2: Primers used to amplify different regions of *Frankia* genome. (Primer numbers for rRNA genes are as per *E.coli* numbering (Embley *et al*, 1988) and for *nif* genes are as per *K.pneumoniae* numbering (Normand *et al*, 1988)).

Sl.#	Target Regions	Primer sequences	References
1.	*nif* HD-IGS	FGPH-750-5'GAAGACGATCCCGACCCCGA3' FGPD-826'-5'TTCATCGACCGGTAGCAGTG3'	Simonet *et al.*, 1991
2.	Distal part of 16S rRNA gene	FGPS-989ac 5'GGGGTCCGTAAGGGTC3' FGPS-1490'-5'AAGGAGGGGATCCAGCCGCA3'	Bosco *et al.*, 1992 Normand *et al.*,1996
3.	Middle part of 16S rRNA gene	FGPS-485-5'CAGCAGCCGCGGTAA3' FGPS-910-5'AGCCTTGCGGCCGTACTCCC3'	Normand *et al.*,1996
4.	Proximal part of 16S r RNA gene	FGPS-6-5'TGGAAAGCTTGATCCCTGGCT3' FGPS-505'-5'GTATTACCGCGGCTGCTG3'	Normand *et al.*,1996

Table3.3: PCR mix

Component*	**Amount**	**Final Concentration**
Water	30.4µl	
10X PCR Buffer	5µl	1X
10mM dATP	1µl	200µM
10mM dCTP	1µl	200µM
10mM dGTP	1µl	200µM
10mM dTTP	1µl	200µM
Forward Primer (5µM)	5µl	0.5µM
Reverse Primer(5µM)	5µl	0.5µM
Taq Polymerase (5U/µl)	0.6µl	0.06U/µl

* All the components are from Sigma Chemical Co., USA.

TABLE: 3.4 List of Restriction enzymes used after cloning and their reaction conditions:

Restriction enzyme with Cat# (Genei,India)	Sequence	Reaction Volume	Reaction temp. (°C)	Amount of RE Used
NcoI (MBE 15S)	C↓CATGG	20µl	37	5units
PstI (MBE 12S)	CTGCA↓G	20µl	37	5units
ApaI (MBE 17S)	GGGCC↓C	20µl	37	5units

Table3.5: List of Restriction enzymes used in present RFLP study

Restriction enzyme with Cat# (Genei,India)	Sequence	Reaction Volume	Reaction temp. (°C)	Amount of RE Used
*Alu*I (MBE-17-S)	AG↓CT	20µl	37	5units
*Taq*I (MBE-7S)	T↓CGA	20µl	65	5units
*Hinf*I(MBE-21S)	G↓ANTC	20µl	37	5units
*Hae*III(MBE-10S)	GG↓CC	20µl	37	5units
*Hha*I (MBE-14S)	GCG↓C	20µl	37	5units
*Mbo*I (MBE-27S)	↓GATC	20µl	37	5units
*Msp*I (MBE-31S)	C↓CGG	20µl	37	5units
HindIII (MBE-6S)	A↓AGCTT	20µl	37	5units
*Eco*RI (MBE-3S)	G↓AATTC	20µl	37	5units

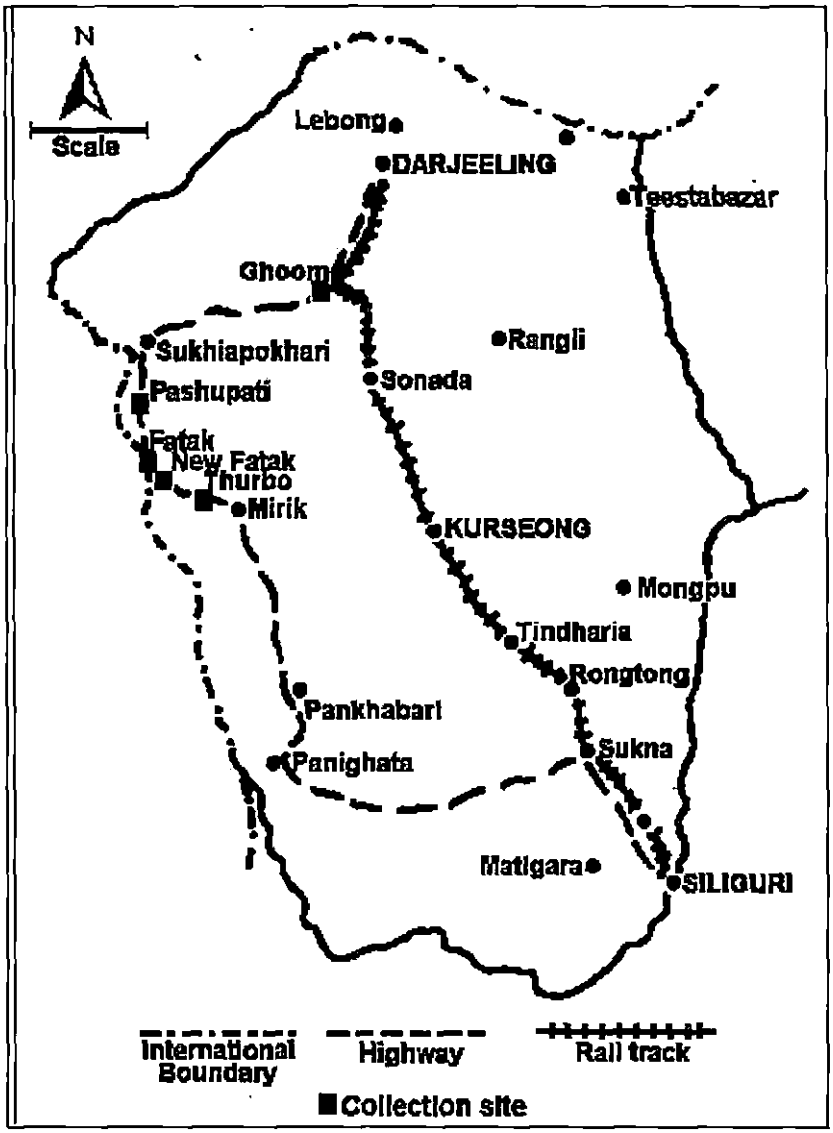

Figure 3.1: Map of Darjeeling District showing collection sites. Scale corresponds to 10 KM.

COLLECTION DATA SHEET

SAMPLE NO. K-2

GENERAL INFORMATION

1. Collection site and state : ST. Alphonsus School
2. Date : 29/2/2000 2A. Time: 2.00 p.m
3. Scientific name: Alnus nepalensis 4. Local name : Uotis
5. Nodules : (Present) / absent
6. Nodules collected : (Yes) / No
7. Root collected : Yes / (No)
8. Seed 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. Rainfall Seasons : From ..... to .....
2. Altitude : 1599 Feet / metre
3. Temperature : (a) Soil: 12 °C (b) Air: 16 °C
4. Topography : Swamp / Plain / (Hilly) / Mountain / Others.
5. Vegetation type : Natural forest / Road side / (Social forest) / others.
6. Management : Cutting / Burning / Natural / (Habitat preserved)
7. Associated species : .....

D. SPECIFIC COLLECTION SITE

1. Site cover : Bare upto 20 % / 21- 40 % / 41- 60 % / (61-80 %) / 81- 100 %
2. Soil pH : 6.2
3. Colour : Red / Yellow / Brown / Grey / Reddish brown
4. Drainage : Flodded / Poorly drained / (Well drained)

F. NODULES

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

COLLECTED BY : Balwinder Bajwa **SIGNATURE** 29/2/2000

Figure 3.2: Sample datasheet of Darjeeling for collection of *Alnus nepalensis* root nodules in the field.

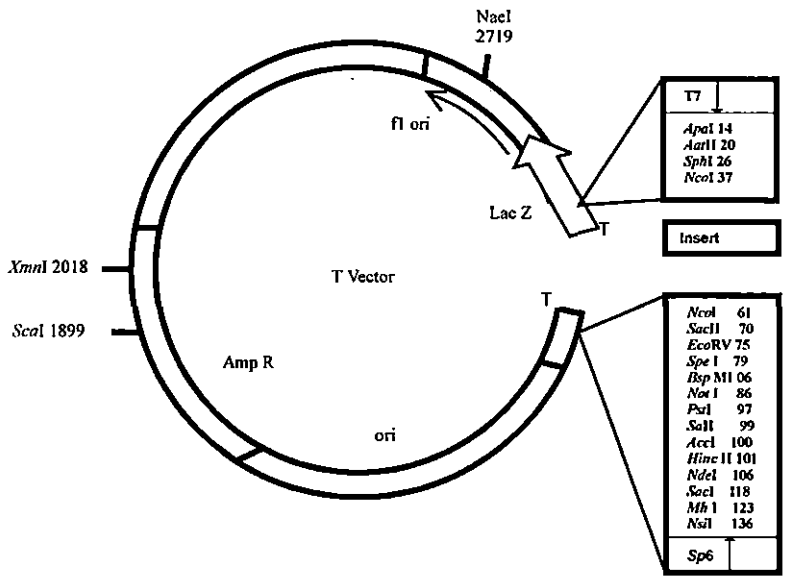

Figure 3.3: Diagram showing T-Vector used in cloning. (Supplied by Genei,India)

CHAPTER 4

Results and Discussion

4.1. Isolation of endophyte

Actinorhizal root nodules are perennial structures which can be described as secondary roots, deformed by the presence of the endosymbiont, *Frankia.* The endosymbiont enters the roots through the root hairs and induces cell division in the root tissue.

Nodules were found copiously in different sites of Darjeeling, pH of the soil was analysed in the range of 6.5-6.8. The size of the clumps of nodule lobes varied from 1-12cm. The colour of the nodules was light brown to reddish brown in its developing stage and later turned into dark brown. Root hairs were present on the nodules and showed branching associated with deformation.

Since nodules were collected from soil and soil contains other microorganisms as well, thus surface sterilization of nodules is a prerequisite for isolation of *Frankia* from root nodules. In the present study among the two surface sterilants tried, H_2O_2 gave the best results. But sterilization is not the only problem encountered in *Frankia* isolation. The second most important step is the removal of epidermal layer of nodule that may otherwise become an unavoidable interruption in isolation steps. So, the epidermal layer of nodule was also peeled off and the tissue was chopped which gave best results in the process.

Frankia is a slow growing organism and only a few slices produced *Frankia* colonies (Plate IV). Each colony originated from one unit that is called as positive cells (Diem and Dommergues 1983). These are referred to as UFF (Units able to form *Frankia*) (Diem et al. 1982). Each nodule slice bears a very limited number of these units. The term UFF was defined broadly to refer either to specific structures or simply to hyphae or clusters of hyphae able to grow out of the nodule. The clusters of hyphae (preferably in the form of nodule slices) may be more suitable for initiating colonies than finely fragmented structures (crushed nodules0, as the new growth of *Frankia* originated from clusters of hyphae (Berry and Torrey 1979). The amount of UFF in the nodule may be related to the age the nodules, its physiological stage like pO_2, soil water content or host plant *Frankia* genomic determinates. In my case I

found that isolation of *Frankia* from fresh as well as carefully preserved nodules (-20°C) gave good results.

The medium used in isolating new *Frankia* strain is critical for success, but no universal or selective media has yet been devised. Several general media for isolation and growth have been reported. Those that have proven effective, range from simple tap water agar to "defined propionate media" and the complex Q- mod media of Lalonde & Calvert (1979)

More than 20 different media are described in the reviews by Baker (1989) and Lechevalier (1990) and many more recipes can be found in the literature. Most of the media proposed for isolation of *Frankia* are supplemented with yeast extract and other diverse compounds believed to promote *Frankia* growth e.g. alcoholic extract of lipids (Quispel and Tak 1978), vitamin (Baker and Torrey 1979) and Lecithin (Lalonde and Calvert 1979).

I found that *Frankia* from *Alnus* root nodules were best isolated in DPM medium, Table 4.1(See table 4.1 at the end of this chapter) lists the isolates obtained.

4.2. Plant Infectivity test

Plant infectivity tests of *Alnus nepalensis* seedlings grown in PLANTON™ plant tissue culture container showed that 50-60% of the plants formed 1-3 nodules within 3-4 weeks. Plants, without young and developing root hairs, hardly nodulate. This indicated that the presence of root hair is a prerequisite for nodulation.

Root hair deformation took place within 1-3 days of inoculation. After nearly 2-3 weeks prenodules were seen .There were 4-5 prenodules per seedling but only 1-2 of them ultimately developed into complete nodules which were visible five weeks after inoculation (Plate V). They were brownish in colour and 1-1.5mm in diameter. On the other hand, negative controls (uninoculated seedlings) showed stunted growth in comparison to inoculated seedlings and at no stage of the experiment were nodules formed in them. Moreover chlorosis also occurred in them after some period. These results confirm the ability of isolates to induce efficient nodules.

4.3. Nitrogenase activity of *Frankia* in pure culture

All the four cultures (AnTB1& AnTB2 and AnGM2 & AnGM5) showed nitrogenase activity of nano mol. of C_2H_2 reduction /mg/hr were in the range of 4.97-5.31 and the differences are statistically insignificant (See Figure 4.1 at the end of this chapter)

4.4. Post isolation workup

After the confirmation of isolates to be *Frankia* both through microscopic observations as well as plant infection tests, best medium for maintenance and growth of the organism isolated must be chosen. It is well known as well as personally observed that *Frankia* grows and prefers nitrogen sources like nitrate or ammonia. But the experiments performed in the present study required cultures grown in nitrogen free media. Thus isolates were cultured in nitrogen free defined propionate minimal medium. And they grew well in DPM medium for carbon source like propionate present in it.

To overcome possible contamination during isolation and sub culturing, Cyclohexamide (Actidion) and Nalidixic acid were added to the DPM. Growth of *Frankia* was found to be normal in the presence of these two additives.

4.5. Structure and ultra-structure of *Frankia*

Light microscopic studies of *Alnus nepalensis* root nodule sections showed characteristic features like vesicle clusters. Each nodule lobe consisted of an outer epidermis and a thick cylinder of cortex within which a single layer of endodermis surrounding the vascular cylinder or stele was present. The isolated colonies in pure culture as seen under light microscope were cushion like and appeared to be embedded in mucilaginous material. Spherical vesicles were present on the periphery of the colony. The average colony was found to be 300µm (Plate IV).

After one week of inoculation root hair infection was studied. When observed under a Light microscope 50-60% of the root hairs were infected within one week.

When studied under scanning electron microscope, typical root hair curling, production of hook like structure and root hair branching was found. Most probably the curling of root hair is due to the entrance of endophyte.

Scanning electron micrographs of pure culture of *Frankia* (plate IV) shows hyphae, vesicles and sporangia. The hyphae were branched and ranged in width from 0.65 to 0.70 µm. When sections of the nodules, both induced as well as field collected, were examined, it was full of vesicles and hyphae were hardly seen (Plate IV). *Frankia* can be more highly differentiated on the basis of its morphological structures. Vesicles are formed at the ends of hyphae. In culture, they are generally globose, but in host cells vary in shape in a manner which appears to be host determined, from filamentous to globose, with or without septa. In the present study vesicles of *Alnus* were observed to be spherical with septations and were present on the periphery of host cytoplasm in the infected cell. Vesicles are electron dense (which enables filamentous shaped ones to be distinguished from hyphae) and have walls which are thickened with as many as 50 monolayers of lipid. The extent of wall thickness is related to oxygen concentration and the walls protect the nitrogenase, which is formed inside the vesicles, from oxygen inactivation (Parsons *et al.* 1987). The length of vesicles ranged between 2.47 to 2.07µm and their diameter ranged from 1.33 to 1.47µm. Small stalks connecting the vesicles with the hyphae were also present. The length of the stalk ranged from 0.97 to 1.33µm and diameter from 0.33 to 0.46µm. *Frankia* usually produces multilocular sporangia which is unique to the *Frankia* group In *Alnus* the sporangia is sporadic and site specific (Torrey 1987; Schwintzer 1990).

Large sporangia were observed in isolates with masses of spores. The length and diameter of the sporangia varied from 9.1to10.3µm and 3.86 to 4.28µm respectively. Evidence to date suggests that all *Frankia* strains have the potential to form sporangia in culture, although they may not always do so inside nodules. In symbiosis nodules may be sp^+ and sp^- independent of their performance in culture.

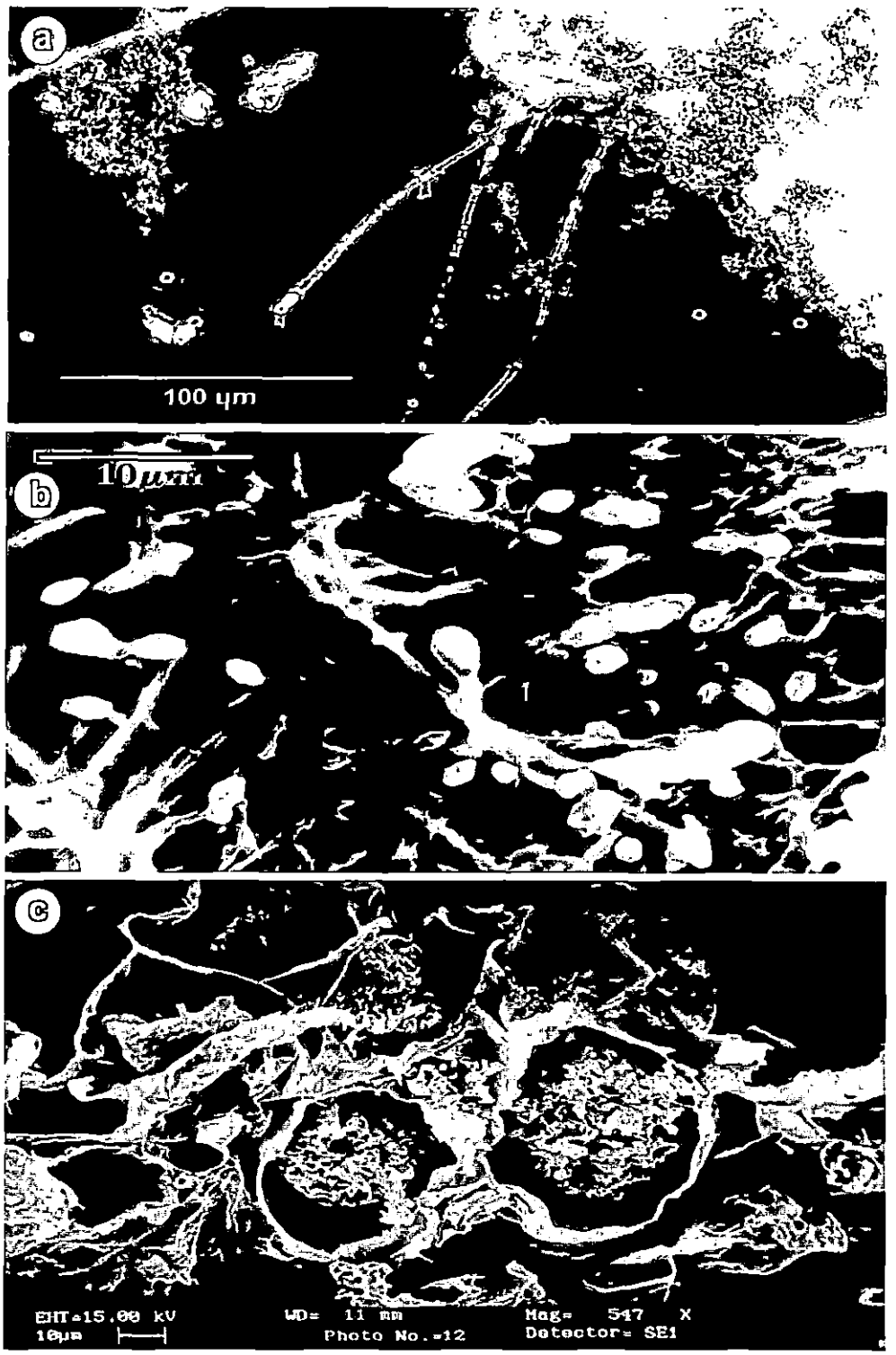

Plate IV

(a) Phase contrast micrograph showing *Frankia* hyphal mass of the isolate (AnTB1). (b) A *Frankial* colony of the isolate (AnTB1) as observed under scanning electron microscope. (c) Scanning electron micrograph of a section of AnTB1 induced nodules showing vesicles inside the cortical cells.

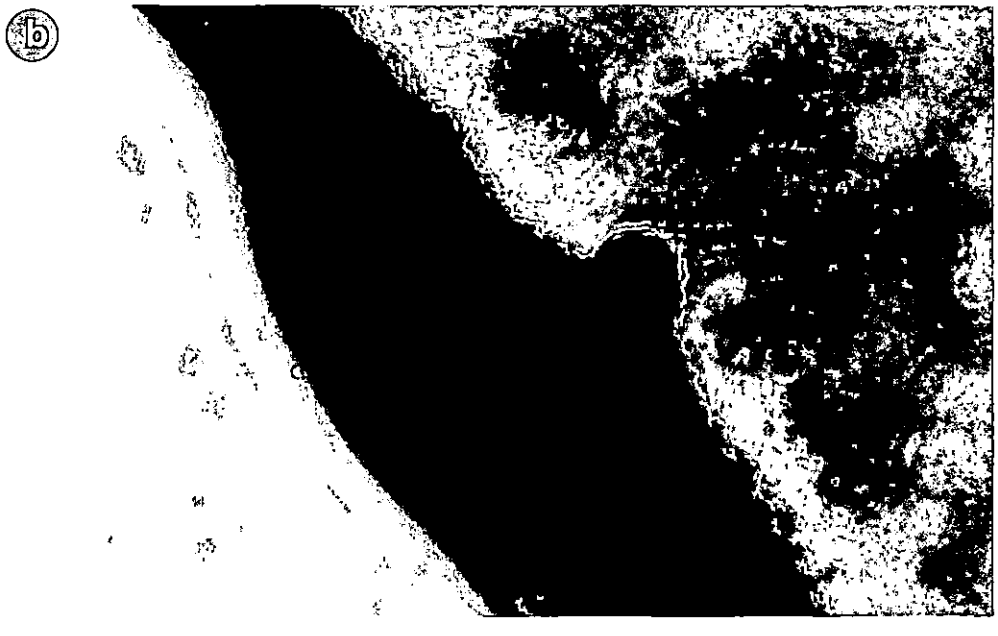

Plate V

(a) Seedlings of *Alnus nepalensis inoculated with Frankia* isolate (An TB1).
(b) A stage of developing nodule as observed under a simple microscope.

4.6. Study of genetic diversity of *Frankia* found in Darjeeling region

Frankia is a sluggishly growing endophyte in culture. There is need of an efficient lysis for a good yield of total genomic DNA. Various methods are employed for proper lysis like chemical dissolution of the cell wall by hot lauryl sulfate (SDS) (Normand *et al.* 1983), use of enzyme like lysozyme and achromopeptidase (Simonet *et al.* 1984) and mechanical shearing of cell wall by ultra-sonication etc. In the present study enzyme induced lysis was tried. In the process lysozyme and achromopeptidase alone or in combination were tried for cultures of different ages. The result is presented in the table 4.2 (See table 4.2 at the end of this chapter).

Presence or absence of DNA was checked on agarose gel. It was observed that keeping all other conditions constant, use of 10mg/ml of lysozyme supplemented with a pinch of achromopeptidase gave best results. Achromopeptidase is a crude enzyme extracted from *Achromobactor lyticus* and has been successfully used with lysozyme to release more protoplasts from hyphae of *Steptomyces*. The present findings are in line with those of Simonet *et al.* (1984).

The DNA isolation procedure as described by Rouvier *et al.* (1996) allowed extraction of sufficient DNA from a single nodule lobe. However, the modification that we have made in incorporating the RNase treatment followed by one more purification step allowed us to obtained substantial amount of sufficiently pure DNA for PCR amplification.

The isolated DNA when subjected to PCR amplification most of the sample gave positive results. Different primers that we have used in the present study amplified distal, middle and proximal part of 16S rRNA gene and intergenic spacer region (IGS) of *nif* H-D of *Frankia* (PlateVI).

Amplification of distal region of 16S rRNA gene was done using primer FGPS989ac & FGPS1490'. Primer FGPS989ac originally developed by Bosco *et al.* 1992 is a *Frankia* specific one and amplifies DNA usually from *Alnus* and *Casuarina* host specificity group. The amplification product was measured to be around 521 bp long and the size is typical (Verghese and Misra 2000). It confirms the presence of

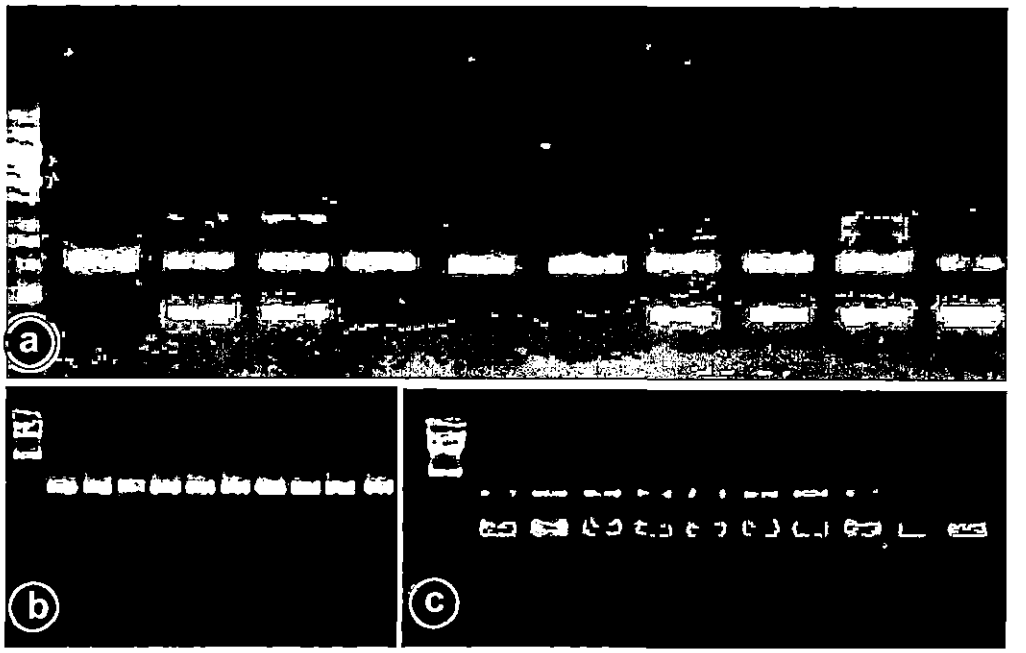

Plate VI

Amplification of DNA isolated from different nodules of *Alnus nepalensis* collected from Darjeeling. The photographs were generated using Kodak digital Science DC-120 Digital Camera.

a : Amplification of distal region of 16S rRNA gene using primers FGPS 989ac and FGPS 1490'. Lane 1, 1 Kb DNA ladder; Lane - 2-11 amplified DNA from different nodules.

b : Amplification of middle part of 16S rRNA gene using primers FGPS 485 and FGPS 910' lane 1, λ DNA *Hind III* digest as molecular marker; lane 2-11- amplified DNA from different nodules.

c : Amplification of *nif*H-D IGS region using primers FGPH 750 and FGPD 826' as in (b) lane 1, λ DNA *Hind III* digest as molecular marker ; lane 2-11 amplified DNA from different nodules.

Frankia in the nodules. All 50 nodules collected from 10 plants of various location of study site amplified.

The proximal and middle part of the 16S rRNA gene was amplified using primers FGPS6 & FGPS505' and FGPS485 & FGPS910' respectively. In both the cases expected sized bands of 540bp and 415bp were observed. However in few cases for instance DNA isolated from nodules could not be amplified with primers FGPS6 & FGPS505' despite of several trials. This indicates that there are major nucleotides substitutions remain in this particular region of the DNA.

In case of middle part of the 16S rRNA gene in all the cases amplification took place. Initially we kept the annealing temperature at 55°C and got more than one bands in place of expected one. As per Verghese & Misra (2000) this may be due to the amplification of the plant DNA along with the *Frankia* DNA. When more stringent (annealing temperature raised by 2°C) single bands were obtained and all the bands were of 415 bp. Now, there is a possibility that this amplification product could be either of *Frankia* origin or plant origin of chloroplast 16S rRNA gene. Therefore, it was further tested by digesting the amplification product with *Eco*R I, since this region of chloroplast DNA does not have an *Eco*R I site. Our products were digested well with EcoR I and produced 155 bp, 135 bp and 125 bp fragments. (PlateVII)

Besides this, the *nif* H-D inter-genic spacer (IGS) region was amplified with primers FGPH-750 & FGPD-826'. However unlike Verghese and Misra who have got multiple bands, we got single band of around 900bp long.

All the PCR products discussed above were subjected to restriction digestion with various restriction enzymes (See Table3.5 at the end of chapter 3). High level of polymorphism was found in distal and proximal parts of 16S rRNA genes and *nif* H-D IGS region (PlateVII). These data were further analyzed with the help of UPGMA based software NTSYSpc (Rohlf 1994) (See Figure 4.2 at the end of this chapter).

Analysis of variance of image of *Frankia* 16SrRNA gene and *nif* H-D IGS region showed that significant difference exists between nodules collected from different parts of Darjeeling hills. Nodules collected from two sites from Fatak (AnF1

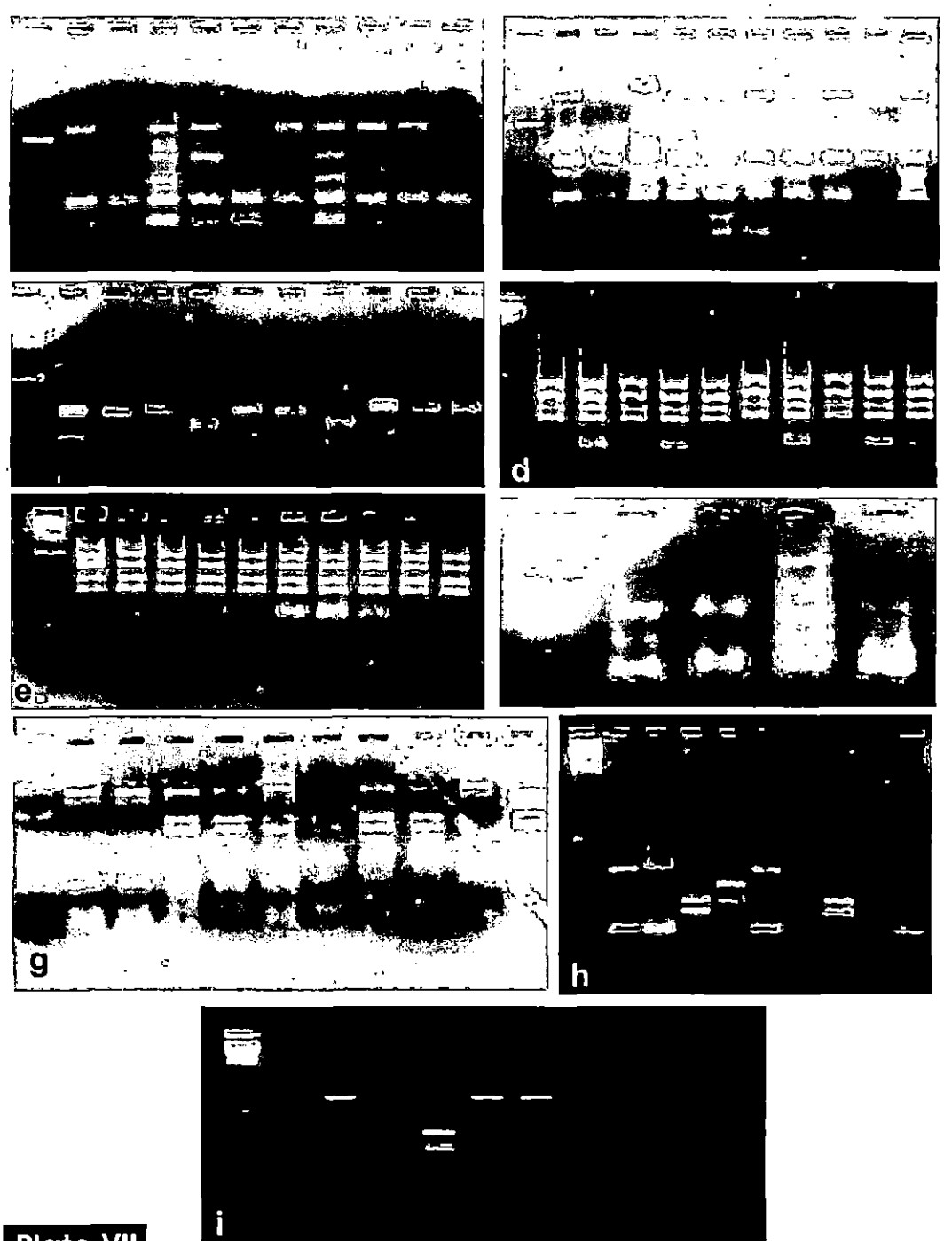

Plate VII

Restriction digestion analysis of DNA isolated and amplified from different nodules of *Alnus nepalensis* of Darjeeling. Lane 1) in all gels contain λ DNA *Hind* III digest as a standard weight marker.

Lanes 2-11 of a, b, & c showing distal part of 16S rRNA gene digested with enzyme *HinfI, Taq* I *&* *Hpa* II respectively

Lanes 2-11 of d&e shows middle part of 16S rRNA digested with enzymes *Hinf* I & *Eco*RI respectively.

Lanes 2-4 of 'f' shows proximal part of 16S rRNA digested with enzyme *Alu*I.

Lanes 2-11 of g, h & i showing *nif* H-D IGS region digested with enzymes *Alu*I, *Taq*.I *&* *Hha*I respectively.

& AnF2) were identical, whereas nodules collected from other sites of Fatak are distantly related to the above two nodules and closer to the nodules of one site of Thurbo. On the other hand AnT3 is close to AnF1 & AnF2. Nodules from Ghoom region i.e. AnG & AnG1 are not very closely related.

Similarly, we have obtained pure cultures from four different trees of two different regions, two from Thurbo and two from Ghoom. However, all four cultures are identical in RFLP pattern and are similar to one Thurbo nodule (AnT3) (Figure 4.2).

We tried to correlate these results with the altitude i.e. whether the genetically close *Frankia* strains belong to more or less same elevation; we found that there is no much correlation. For example, nodules found in Ghoom at an altitude of 7407ft. are close to the nodules found in New Fatak region (altitude ~ 3000 ft.), whereas nodules from different parts of Fatak located at same altitude are not so close. Likewise, cultures from Thurbo and that of Ghoom are 100% identical whereas the altitudinal difference of Ghoom and Thurbo is around 1800 ft.

Therefore from the present study it is found that the method of PCR-RFLP could detect polymorphism among different *Frankia* genotypes and considerable variation exists among *Frankia* of Darjeeling hills. The distributions of *Frankia* genotypes are not dependent on the altitude of the sites.

4.7. Cloning of PCR products

Since a high level of polymorphism was detected in the samples as discussed above we decided to go for cloning of the PCR products. For this purpose we have digested the PCR product with different restriction enzymes as mentioned in material and methods (See Section 3.12.5.). And we have got single bands separated from the plasmid and the bands are of expected sizes, 521bp (in case of distal part) and around 900bp in case of *nif* H-D IGS region (See Figure 4.3 at the end of this chapter).

4.8. Study of host's influence on nitrogenase activity of '*in-nodule' Frankia.*

In the present study our aim was to study whether it is the *Frankia* who decides the amount of nitrogen to be fixed or it is the host that effects the production of overall nitrogen fixed during the nitrogen fixation process. It is otherwise very difficult to prove but by performing the two experiments simultaneously a little conclusion could be drawn.

The first attempt in this study was an Acetylene reduction assay of the nodules collected from a forest stand of Alder in Gangtok. ARA results of these nodules AnG1, AnG2, AnG3, AnG4, AnG5, AnG6, AnG7, AnG8, AnG9 and AnG10 collected from 10 different trees showed that there was a lot of difference in the amount of Nitrogen fixed by each one of them (See Figure 4.4 at the end of this chapter).

Next a study was performed on RFLP of the same set of nodules. As mentioned in (See Section 3.6.2) the genomic DNA was first isolated and when it was subjected to PCR amplification with four set of primers (SeeTable 3.2 at the end of chapter 3) it was observed that all primers other than the primers used for amplifying the *nif* HD-IGS region produced a positive result producing the bands in the gel of the expected size respectively (Plate VIII). Only the one pair of primers used for amplifying the *nif* HD-IGS region, FGPH750 & FGPD826' gave negative result despite of the several trials i.e. it could not amplify the region. It may be due to some major nucleotide substitutions in that particular region. Restriction digestion was carried out as mentioned in the material and method (Section 3.13) (Plate VIII). The PCR RFLP data were scored as present (1) or absent (0) using the SIMQUAL program and DICE coefficients. A matrix was computed and phonograms developed by a UPGMA clustering of the matrix NTSYS-pc (Rohlf 1994) (Figure 4.5 at the end of this chapter).

Therefore it is apparent from the above result that *Frankia* strains belonging to AnG1, AnG2, AnG6 and AnG7 are from same genomic group (Rouvier 1996). Now it is expected that the *Frankia* from the same genomic group may fix same amount of

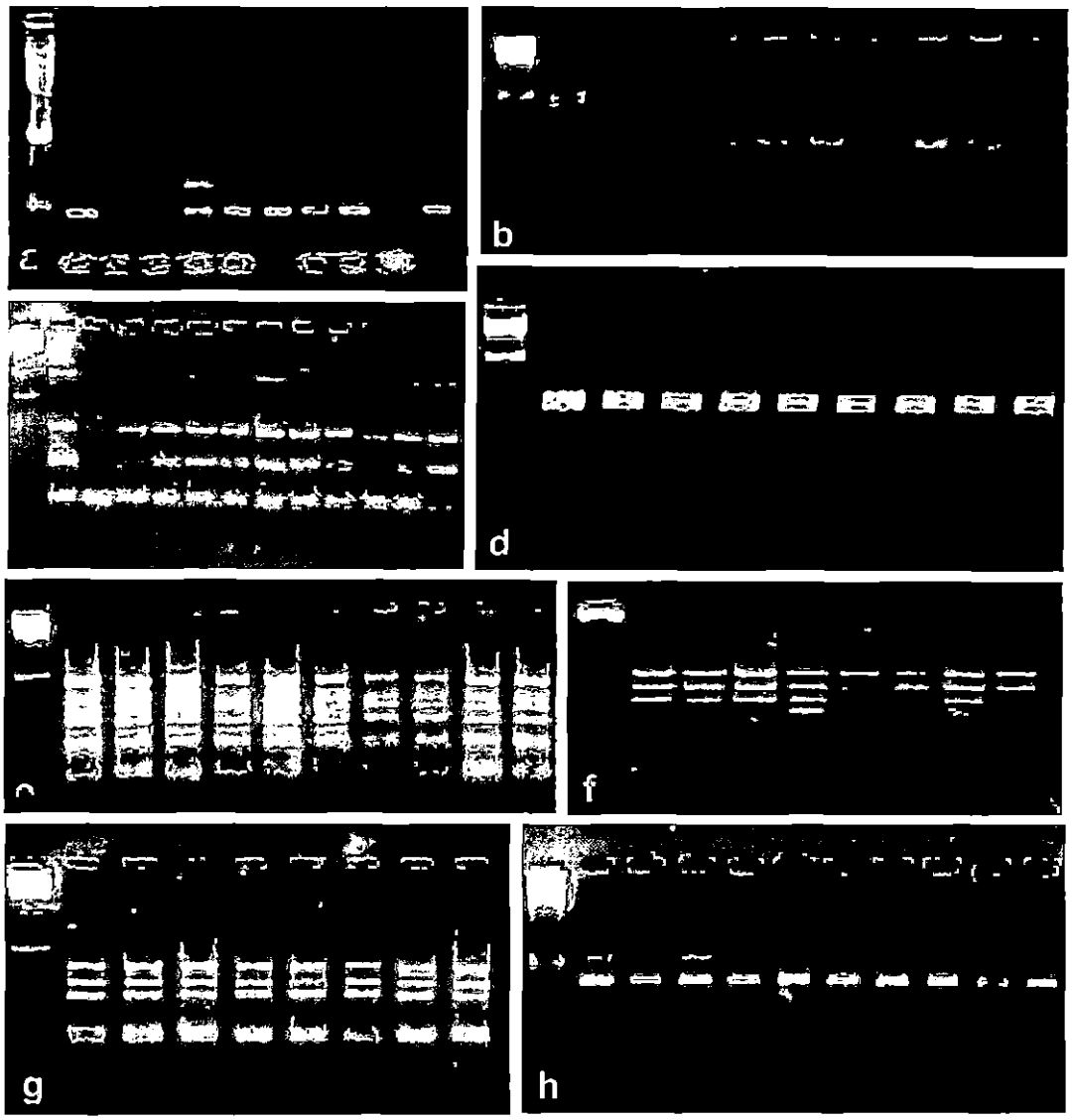

Plate VIII

Amplification and restriction analysis of DNA isolated from nodules of *Alnus nepalansis* collected from Gangtok. Lane 1, in all gels contain λ DNA *Hind*III digest as a molecular marker.

(a) Lanes 2-11 showing amplification of distal region of 16S rRNA gene using primer FGPS 989ac and FGPS 1490'.

(b) Lanes 2-11, shows restriction digestion pattern of distal part of 16S rRNA gene using enzyme *Hae* III.

(c) Lanes 2-13 showing distal part of 16S rRNA gene cut with the enzyme Taq.*I.*

(d) Lanes 2-9 shows amplification of middle part of 16S rRNA gene using primer FGPS 485 and FGPS 910'.

(e&h) Lanes 2-11 showing restriction digestion pattern of middle part of 16S rRNA gene using enzymes *Alu*I and *Hha*I respectively.

(f&g) Lanes 2-9 shows restriction digestion of middle part of 16S rRNA gene using enzymes *Hin*fI and *Taq*I *respectively.*

Nitrogen under other constant parameters (age and height of the host plant, soil temperature, soil pH, moisture content etc.). However our ARA study shows that there are statistically significant differences persist among the above mentioned nodules. For instance AnG1 fixed 11.34µmol of C_2H_4 per gram of fresh weight of nodules per hour whereas AnG2 fixes 22.41µmol of C_2H_4 per gram of fresh weight of nodules per hour. Again AnG10 fixes only 9.22µmol of C_2H_4 per gram of fresh weight of nodules per hour. Since all other parameters are constant we may conclude that the host plant does have a role in influencing *Frankia* in terms of Nitrogen *Frankia* would fix. Future studies therefore should include fingerprinting of host plants and identification of genes involved in nodulation and Nitrogen fixing process.

Table: 4.1: *Frankia* isolates obtained from root nodules of *Alnus nepalensis* collected from various sites of Darjeeling.

Isolates	Plant	Place
AnTB1	*Alnus nepalensis*	Thurbo
AnTB2	*Alnus nepalensis*	Thurbo
AnGM2	*Alnus nepalensis*	Ghoom
AnGM5	*Alnus nepalensis*	Ghoom

Table: 4.2: Lysis of cell wall using enzymes and their effectiveness

Age of culture	Enzymes used	Results
7-15 days	Achromopeptidase	☹
7-15 days	Lysozyme	😐
7-15 days	Lysozyme+acromopeptidase	☺
20-30 days	Achromopeptidase	☹
20-30 days	Lysozyme	☹
20-30 days	Lysozyme+achromopeptidase	😐

Symbols: 😐 denotes lysis; ☺ denotes better lysis; ☹, no lysis.

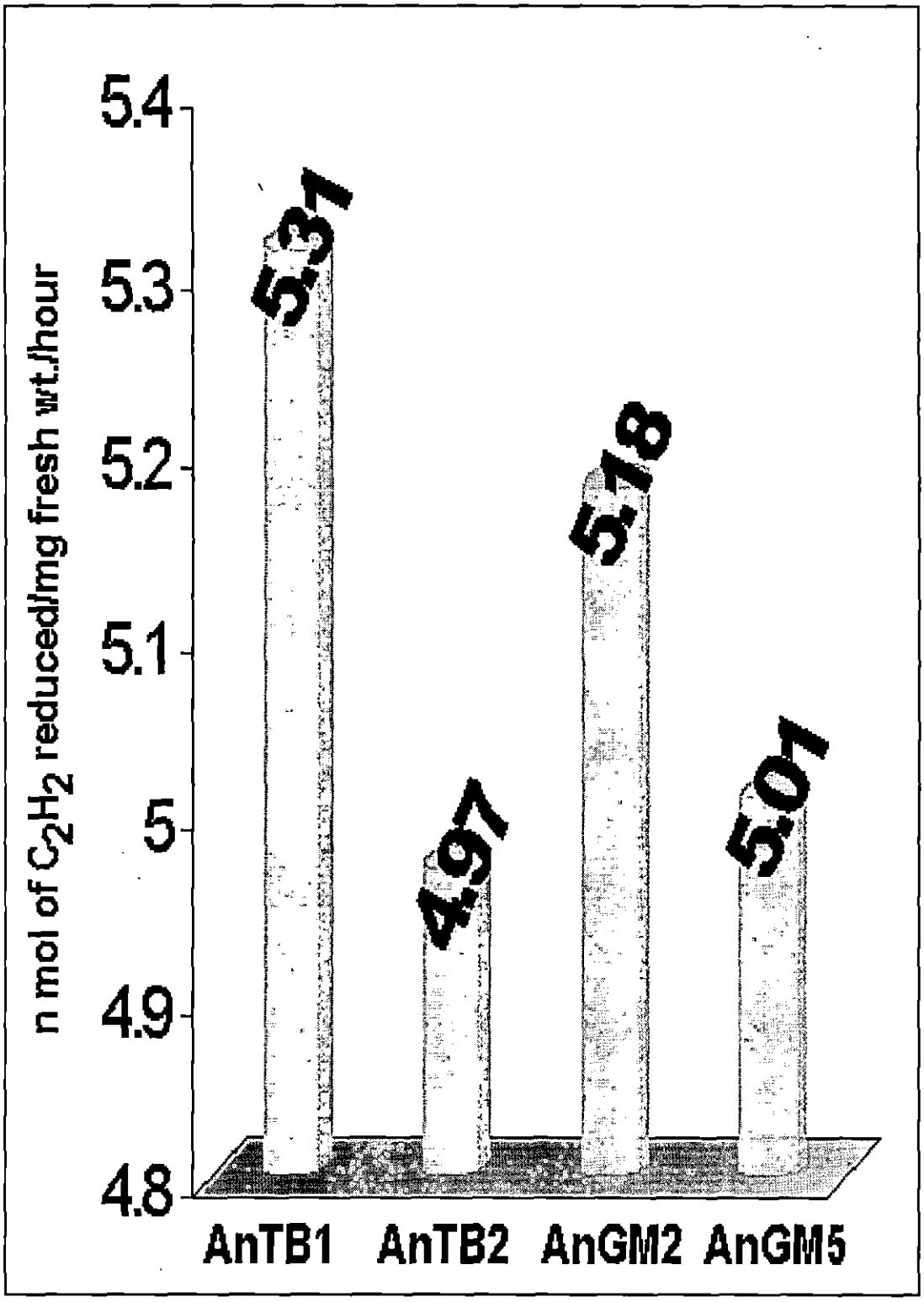

Figure 4.1: Graph showing Nitrogenase activity of *Frankia* isolates from Darjeeling region.

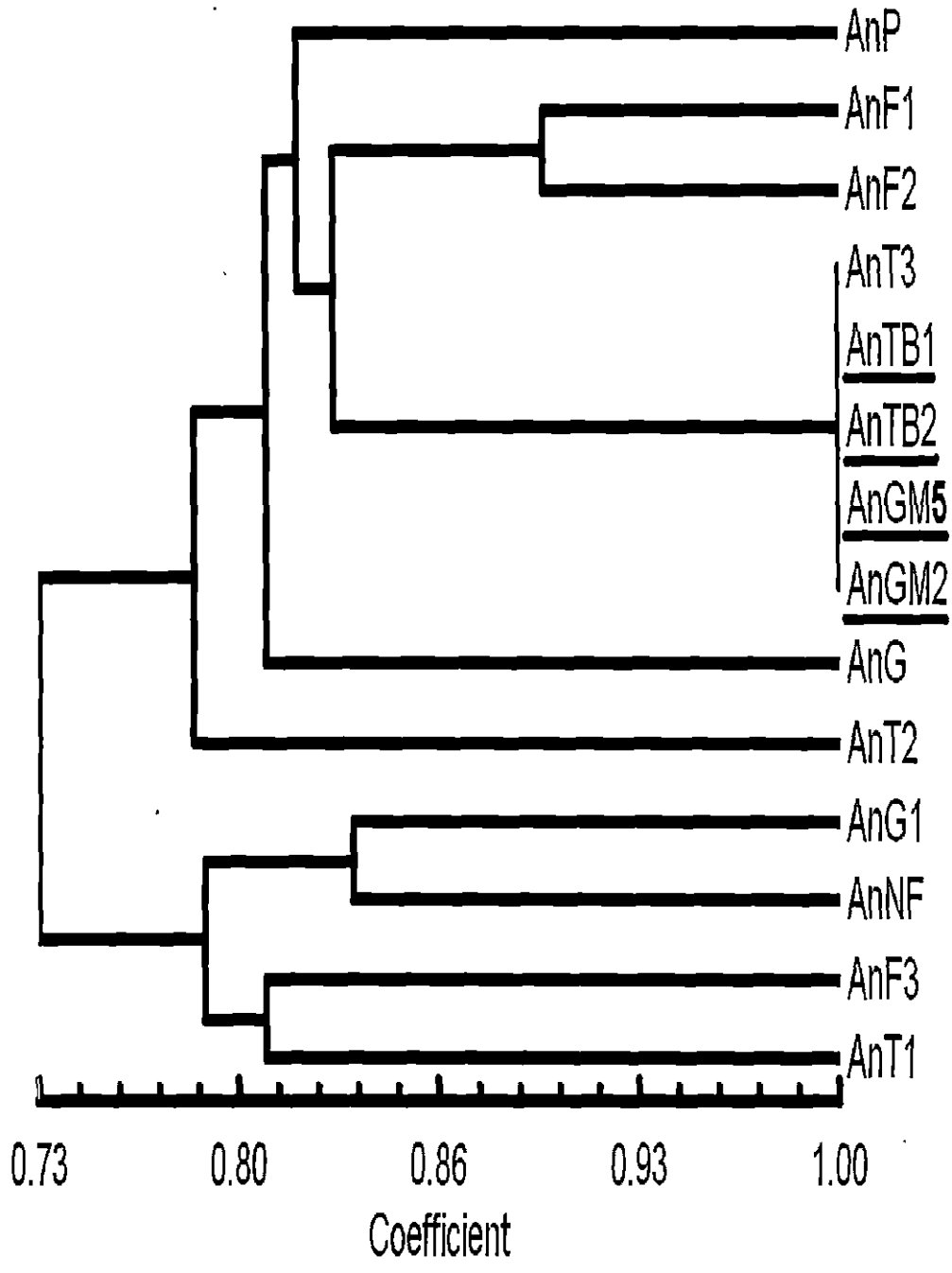

Figure 4.2: Phylogenetic tree based on PCR-RFLP pattern of 16S rRNA and *nif* HD IGS region analysed with UPGMA clustering of the matrix using NTSYSpc (Rohlf, 1994) for cultures and nodules of Darjeeling region.

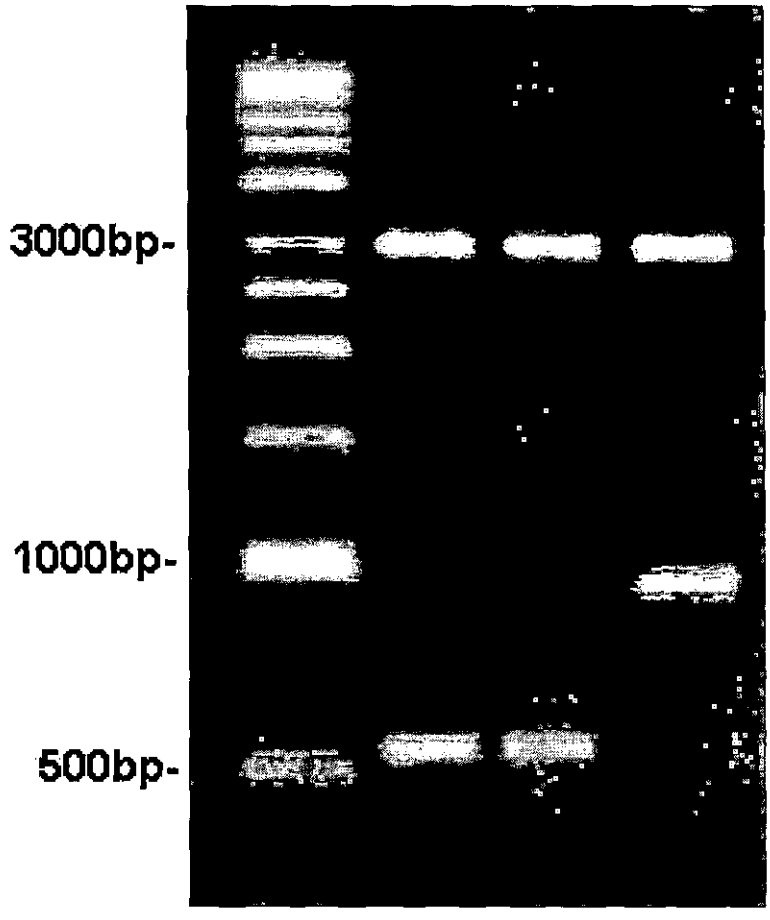

Figure 4.3: Agarose gel showing T-vector. PCR amplified products cloned in T-vector and digested with various restriction enzymes to verify the insert. Lane 1, molecular wt. ladder (Sigma Cat# D3937); Lane 2&3, distal part of 16S rRNA gene (521bp); Lane 4, nif H-D IGS region (~900bp).

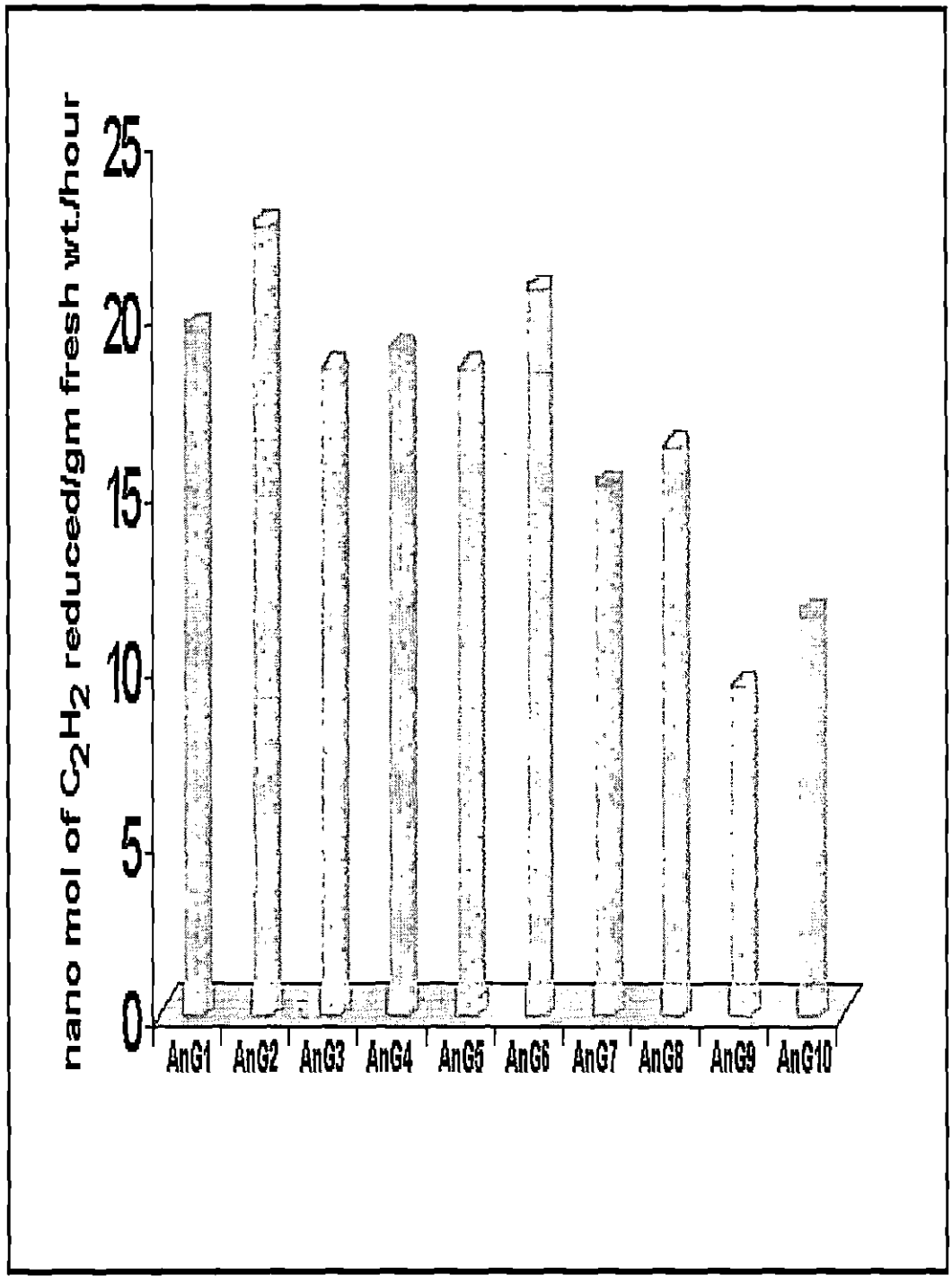

Figure 4.4: Graph showing nitrogenase activity of *Frankia* in nodules collected from different trees of *Alnus nepalensis* found in Tadong, Gangtok

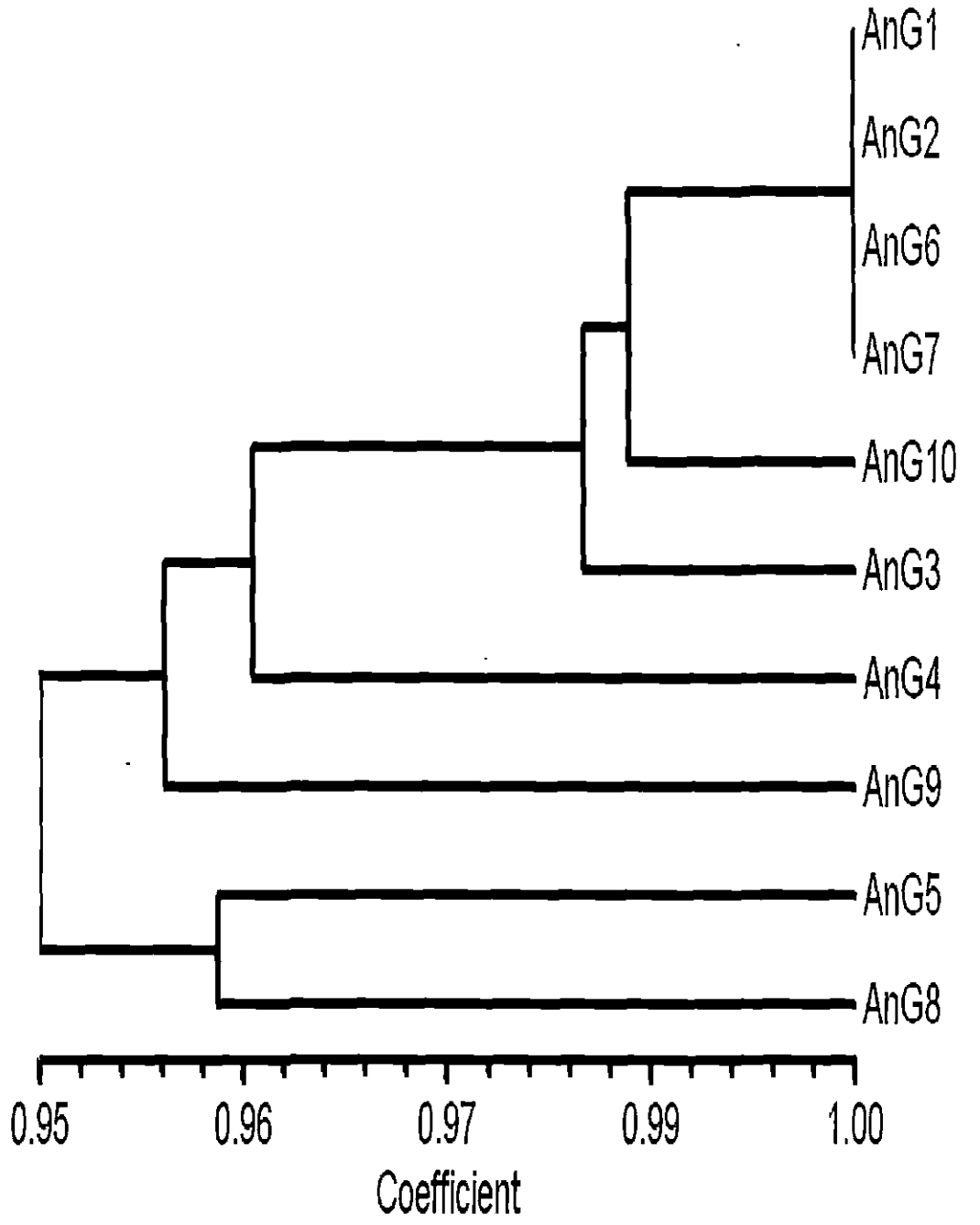

Figure 4.5: Neighbour joining consensus tree for aligned sequences of the distal part of 16S rRNA region of *Frankia* based on the PCR-RFLP pattern and analyzed with UPGMA clustering of the matrix using NTSYSpc (Rohlf, 1994) as seen in nodules collected from different trees of *Alnus nepalensis* found in Tadong, Gangtok.

CHAPTER 5

Conclusion

In Conclusion, the following points are highlighted:

- *Frankia* isolates from nodules of *Alnus nepalensis* were obtained.
- Nodulation tests, morphological and anatomical studies under SEM and DNA amplification using *Frankia* genus specific primers for 16S rRNA gene and *nif* genes confirmed the isolates to be *Frankia*.
- High level of polymorphism was detected in nodules collected from Darjeeling.
- Cloning was done of the PCR product obtained from nodule DNA of Darjeeling sample that showed high level of polymorphism. The clones obtained may be used for further studies.
- Nitrogenase test of the nodules collected from Gangtok when correlated to RFLP studies showed that host does play a role on Nitrogen fixation rate of *Frankia*.

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Appendix

APPENDIX-I

Composition of culture mediums for *Frankia*

- **DPM Medium: (Nitrogen free)**

Constituents	Stock soln. (Per/lit)	Working Soln. (Per/lit)
Macronutrients	10X	
KH_2PO_4[1]	10.0 g	7348µM
$MgSO_4$[1]	1.0 g	0899µM
$Cacl_2.2H_2O_2$[2]	0.1 g	0068µM
$C_3H_5O_2Na$[5]	12.0 g	12490µM
Micronutrients	1000X	
H_3BO_3[1]	2.860 g	46.3µM
$MnCl_2.4H_2O$[4]	1.810 g	9.1µM
$ZnSO_4.7H_2O$[1]	0.220 g	0.8µM
$CuSO_4.5H_2O$[1]	0.080 g	0.3µM
Na_2MoO_4[2]	0.025 g	0.1µM
$CoCl_2.7H_2O$[2]	0.025 g	0.1µM
Iron	1000X	
FeSO4.7H2O[2]	10.008 g	370.4µM
Na_2EDTA[3]	13.410 g	36.0µM

pH-7.00.

1. E MERCK (India) Ltd. Mumbai, India.
2. SRL SISCO RESEARCH LABORATORIES Pvt. Ltd. Mumbai, India.
3. QUALIGENS Fine Chemicals. Glaxo India Ltd. Mumbai, India.
4. HI MEDIA laboratories limited. Mumbai, India
5. SIGMA-ALDRICH chemicals Pvt Ltd, USA

- **F Medium**

Constituents	Stock soln. (Per/lit)	Working Soln. (Per/lit)
Macronutrients	10X	
KH_2PO_4[1]	5.0g	2871µM
$MgSO_4$[1]	2.0g	0811µM
$CaCl_2.2H_2O$[2]	1.0g	2127µM
D-Glucose[5]	100.0g	5550µM
Hydrolysate casein[1]	40.0g	
Yeast extract[2]	0.5g	
Oligo quispel	1000X	
H_3BO_3[1]	1.500g	24.3µM
$MnSO_4.H_2O$[1]	0.800g	4.7µM
$ZnSO_4.7H_2O$[1]	0.600g	2.1µM
$CuSO_4.5H_2O$[1]	0.100g	0.4µM
$(NH_4)_8Mo7O_{24}4H_2O$[1]	0.200g	0.16µM
$CoSO_4.7H_2O$[1]	0.025g	0.09µM
Iron	1000X	
$C_8H_8O_7.H_2O$[4]	10.000g	47.6µM
$C_8H_8O_7Fe.3H_2O$[4]	10.000g	33.5µM
Vitamin	1000X	
Thiamine HCl[5]	0.010g	0.03µM
Nicotinic acid[5]	0.050g	0.40µM
Pyridoxine HCl[5]	0.050g	0.24µM
Tween 80[4]		1ml

pH-7.00

1. E MERCK (India) Ltd. Mumbai, India.
2. SRL SISCO RESEARCH LABORATORIES Pvt. Ltd. Mumbai, India.
3. QUALIGENS Fine Chemicals. Glaxo India Ltd. Mumbai, India.
4. HI MEDIA laboratories limited. Mumbai, India
5. SIGMA-ALDRICH chemicals Pvt Ltd, USA

- **Hoagland Medium: N_2-free**

Constituents	Stock soln. (Per/lit)	Working Soln. (Per/lit)
Macronutrients	10X	
KH_2PO_4[1]	1.36 g	999 µM
$MgSO_4$[1]	3.38 g	3039 µM
Micronutrients	1000X	
H_3BO_3[1]	2.860 g	46.3µM
$MnCl_2.4H_2O$[4]	1.810 g	9.1µM
$ZnSO_4.7H_2O$[1]	0.220 g	0.8µM
$CuSO_4.5H_2O$[1]	0.080 g	0.3µM
Na_2MoO_4[2]	0.025 g	0.1µM
$CoCl_2.7H_2O$[2]	0.025 g	0.1µM
Iron	1000X	
$FeSO_4.7H_2O$[2]	10.008 g	370.4µM
Na_2EDTA[3]	13.410 g	36.0µM

pH-7.00

1. E MERCK (India) Ltd. Mumbai, India.
2. SRL SISCO RESEARCH LABORATORIES Pvt. Ltd. Mumbai, India.
3. QUALIGENS Fine Chemicals. Glaxo India Ltd. Mumbai, India.
4. HI MEDIA laboratories limited. Mumbai, India

- OS-1 Medium

Constituents	Stock soln. (Per/lit)	Working Soln. (Per/lit)
Macronutrients	10X	
KH_2PO_4[1]	1.5g	1102µM
$NaH_2PO_4.2H_2O$[1]	1.3g	833µM
$MgSO_4.7H_2O$[1]	0.5g	202µM
KCl[1]	1.0g	1341µM
$NaC_2H_3O_2.3H_2O$[4]	5.0g	3658µM
EDTA[3]	0.1g	26.8µM
Yeast extract[2]	2.5g	
Tryptone[4]	2.5g	
Tween 80[4]		1ml

pH-7.00

1. E MERCK (India) Ltd. Mumbai, India.
2. SRL SISCO RESEARCH LABORATORIES Pvt. Ltd. Mumbai, India.
3. QUALIGENS Fine Chemicals. Glaxo India Ltd. Mumbai, India.
4. HI MEDIA laboratories limited. Mumbai, India

- Q_{mod}

Constituents	Stock soln. (Per/lit)	Working Soln. (Per/lit)
Macronutrients	10X	
KH_2PO_4[1]	3.0g	1722µM
$NaH_2PO_4.2H_2O$[1]	2.3g	1474µM
$MgSO_4.7H_2O$[1]	2.0g	0811µM
KCl_2[1]	2.0g	1682µM
D-Glucose[5]	100.0g	55506µM
Yeast extract[2]	0.5g	
Peptone[4]	50.5g	
Micronutrients	1000X	
H_3BO_3[1]	1.500g	24.3µM
$MnSO_4.H_2O$[1]	0.800g	4.7µM
$ZnSO_4.7H_2O$[1]	0.600g	2.1µM
$CuSO_4.5H_2O$[1]	0.100g	0.4µM
$(NH_4)_8Mo7O_{24}4H_2O$[1]	0.200g	0.16µM
$CoSO_4.7H_2O$[1]	0.025g	0.09µM
Iron	1000X	
$C_8H_8O_7.H_2O$[4]	10.000g	47.6µM
$C_8H_8O_7Fe.3H_2O$[4]	10.000g	33.5µM
Lipid supplement	10X	
L-∝-Lecithin[5]	0.400g	

pH-7.00

1. E MERCK (India) Ltd. Mumbai, India.
2. SRL SISCO RESEARCH LABORATORIES Pvt. Ltd. Mumbai, India.
3. QUALIGENS Fine Chemicals. Glaxo India Ltd. Mumbai, India.
4. HI MEDIA laboratories limited. Mumbai, India
5. SIGMA-ALDRICH chemicals Pvt Ltd, USA

- **L.B. medium: (Liquid)**

  Bactopeptone-10g/lt.

  NaCl-10g/lt.

  Yeast Extract-5g/lt.

  The above mentioned components were dissolved in 1 litre of double distilled water and then autoclaved.

- **L.B. medium plates: (Solid)**

  Bactopeptone-10g/lt.

  NaCl-10g/lt.

  Yeast Extract-5g/lt.

  Agar-1.2%

  The above mentioned components were dissolved in 1 litre of double distilled water and then autoclaved. After cooling to 40°C

APPENDIX-II

Composition of Buffers:

- **0.2M Cacodylate buffer (1X Concentration)**

  197mM Cacodylic acid

  (Dimethylarsinic acid, $Na(CH_3)_2AsO_2.3H_2O$)

  Sodium Salt.

  Sodium cacodylate (42.2g) was dissolved in 800ml of sterile distilled water. pH was adjusted to 7.4 with HCl and the volume was made upto 1000ml with sterilized distilled water.

- **DNA Extraction buffer (1X Concentration)**

  100mM Trizma base (pH-8.0)

  20mM EDTA (pH-8.0)

  1.4M NaCl

  2% (W/V) CTAB

  1% (W/V) PVPP

  54g of molecular biology grade Trizma base (Sigma, USA. Cat#T-1503, Tris (hydroxymethyl)aminomethane,$C_4H_{11}NO_3$, FW-121.1) was dissolved in 800ml of sterile double distilled water, pH was adjusted to 8.0. It was divided into two parts, to one part 7.44g EDTA was added. In other part 81.82g NaCl,20g CTAB (Hexadecyl trimethyl ammonium bromide, $C_{19}H_{42}NBr$) and 10g PVPP was added.Both the parts were then mixed.

- **DNA Loading Buffer (6X Concentration)**

  (TypeIII, Sambrook et al., 2001)

  0.25% Bromophenol blue

  0.25% Xylene cyanol FF

  30% Glycerol in DD H_2O

  Two and a half grams of Bromophenol blue and Xylene cyanol was dissolved in 1000ml of 30% Glycerol.

- **PBS-Phosphate Buffered Saline (pH-7.4)**

  137mM NaCl

  2.7mM KCl

  10mM Na_2HPO_4

  1.8mM KH_2PO_4

  8gm of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4 and 0.24g 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 upto 1litre with Sterile double distilled water.

- **Peeling Buffer**

  NaCl-8g

  KCl-0.2g

  Na_2HPO_4-1.44g

  KH_2PO_4-0.24g

  The above components were dissolved in 1 litre of double distilled water after adjusting the pH to 7.4 with HCl.To this 30g of Polyvinyl Pyrollidone (PVP) was added and it was autoclaved.

- **TE- Tris EDTA Buffer (pH-8)**

  10mM Tris (pH-8.0)

  10mM EDTA (pH-8.0)

  1.21g molecular biology grade Trizma base (Sigma, USA. Cat#T-1503, Tris (hydroxymethyl) aminomethane,$C_4H_{11}NO_3$, FW-121.1). was dissolved in 400ml of double distilled water and the pH was adjusted with conc. HCl to 8.0 and sterilized by autoclaving. Similarly 0.372g Di-Sodium EDTA was dissolved in 400ml of double distilled water. The solution was stirred properly and the pH was adjusted with NaOH pellets and sterilized by autoclaving. Both the solutions were then mixed and the volume was made upto 1 litre with sterilized double distilled water.

- **TBE- Tris-Borate EDTA Buffer (5X Concentration)**

  0.045M Tris-Borate

  0.001M EDTA

  Preparation of 5X stock:

  54g of molecular biology grade Trizma base (Sigma, USA. Cat#T-1503, Tris (hydroxymethyl)aminomethane, $C_4H_{11}NO_3$, FW-121.1) and 27.5g Boric acid were dissolved in 800ml of sterile double distilled water. To it 20ml of 0.5M EDTA (pH-8.0) was added.

  TBE was used in a final concentration of 1X, so the 5X stock was diluted to 1X.