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.

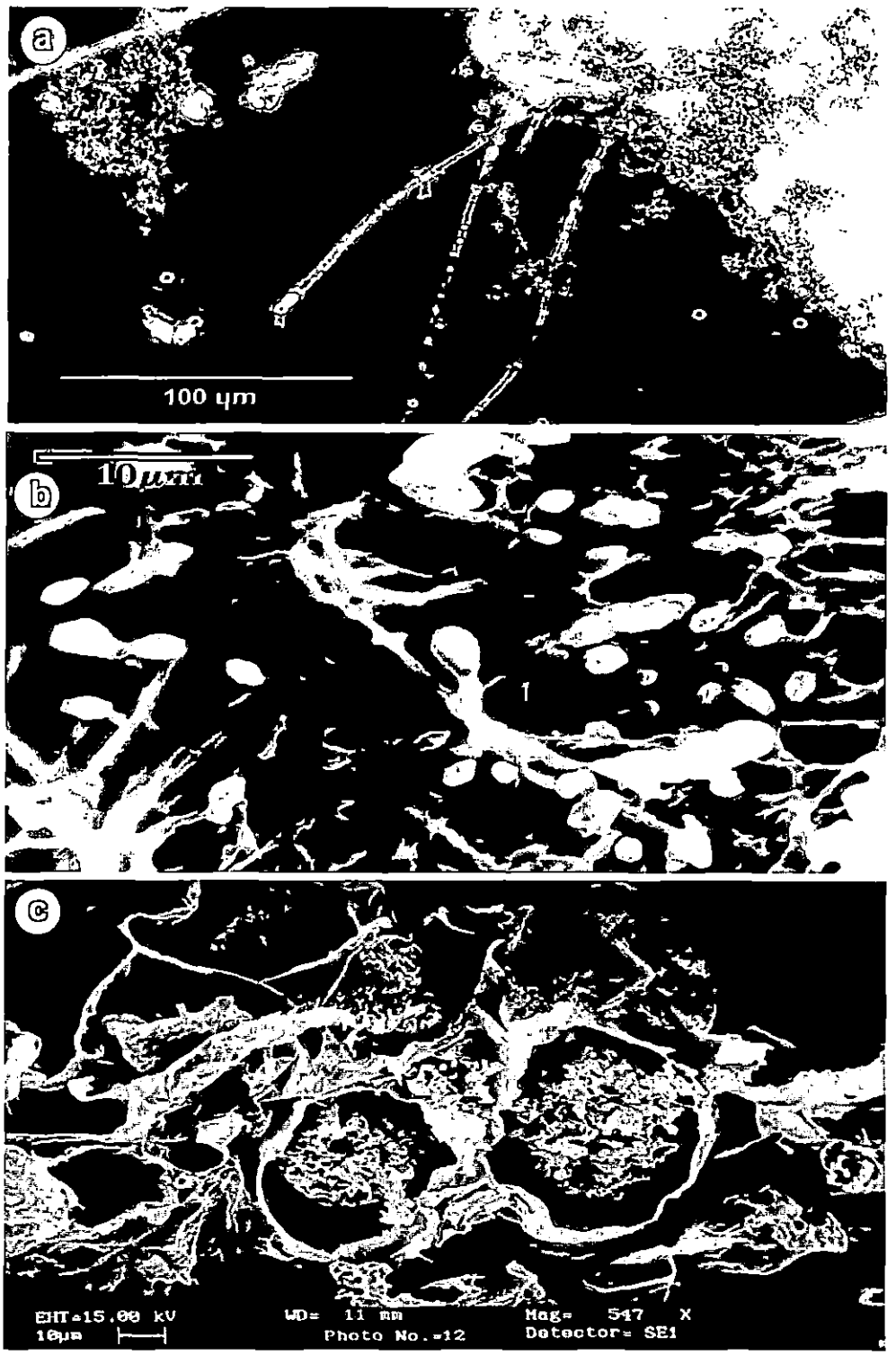

Plate IV

(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.

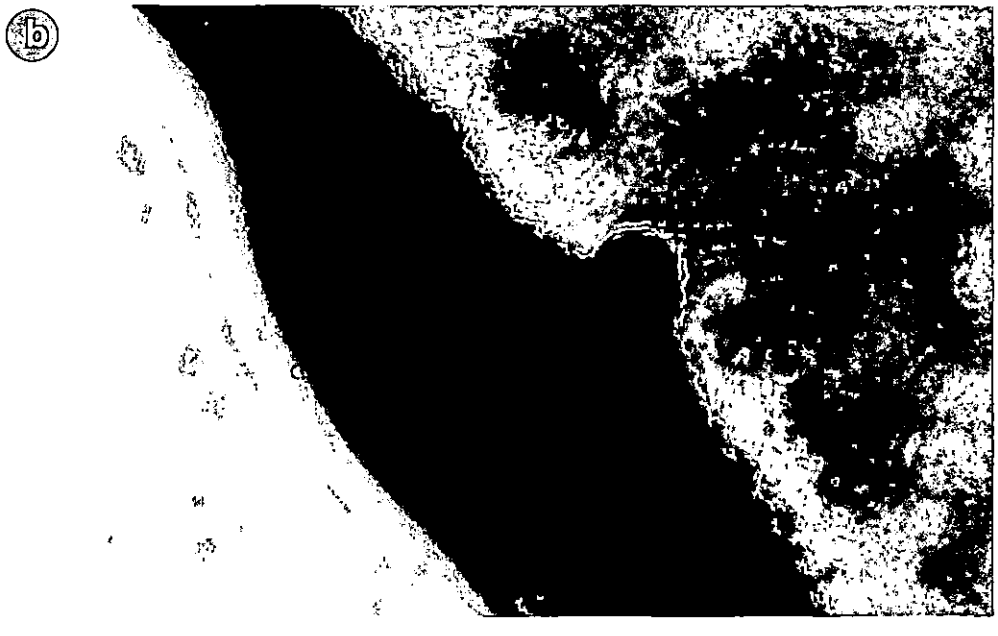

Plate V

(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

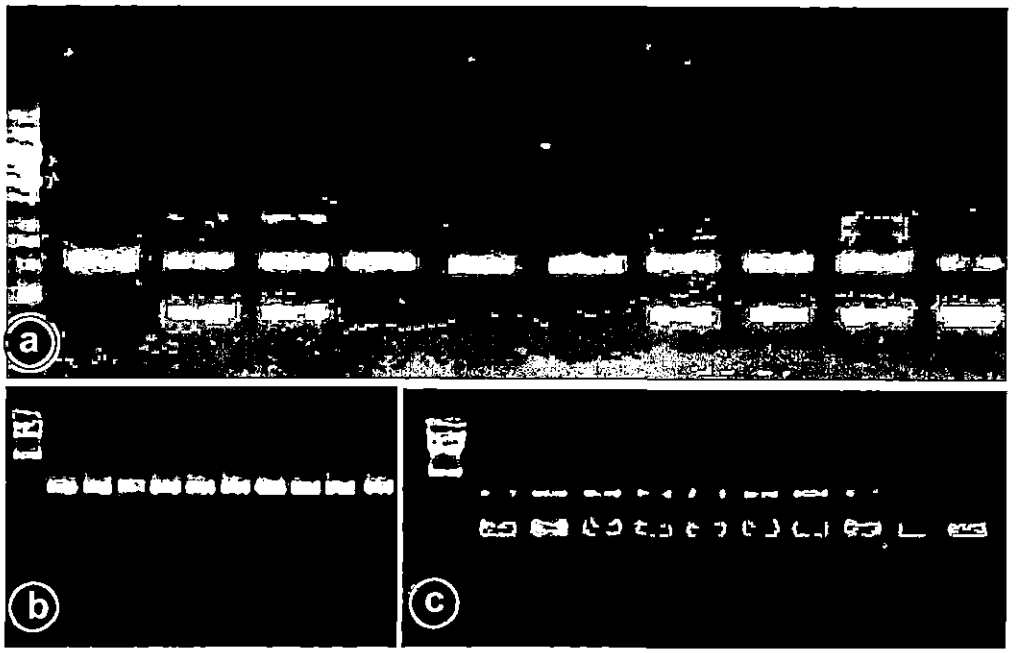

Plate VI

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

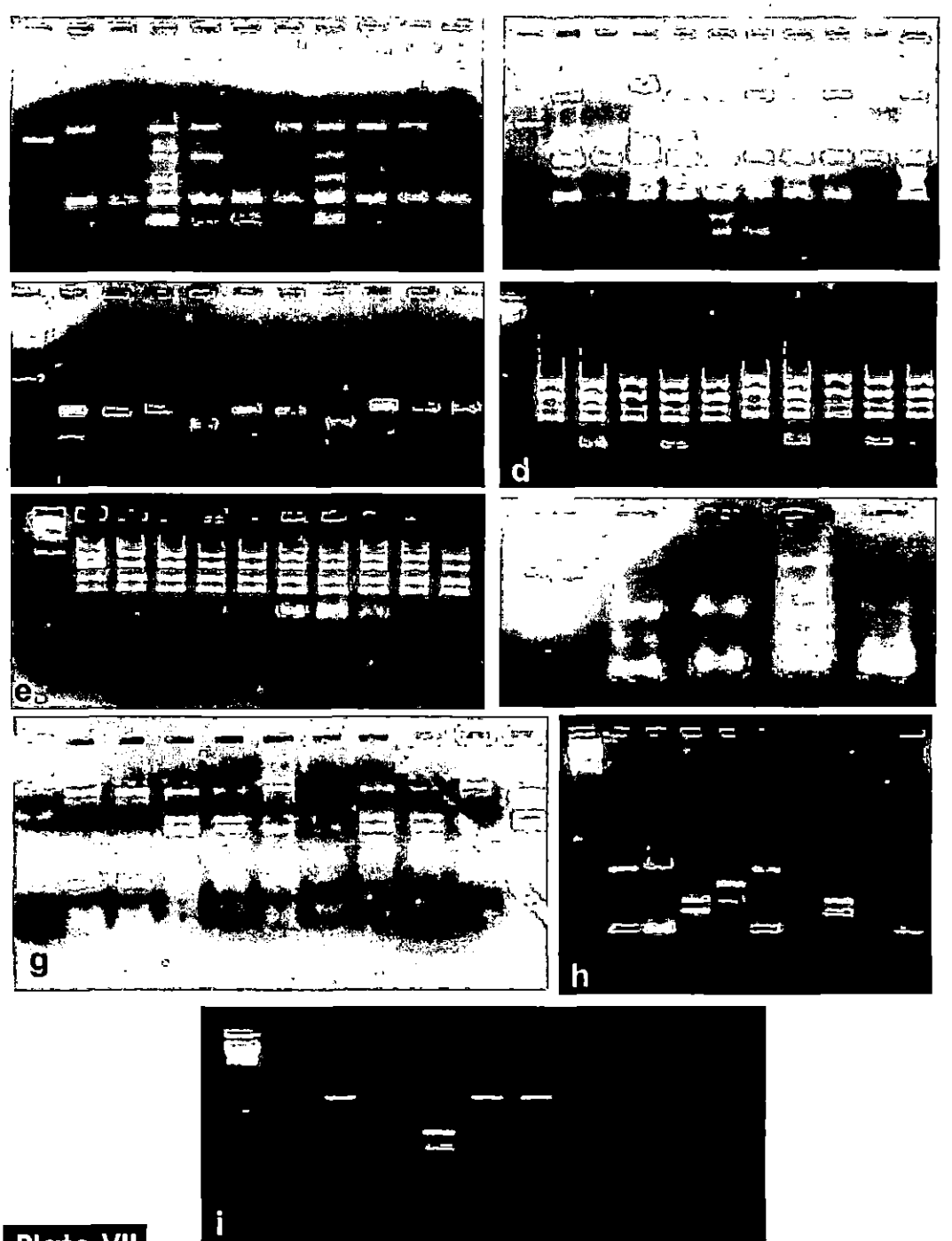

Plate VII

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

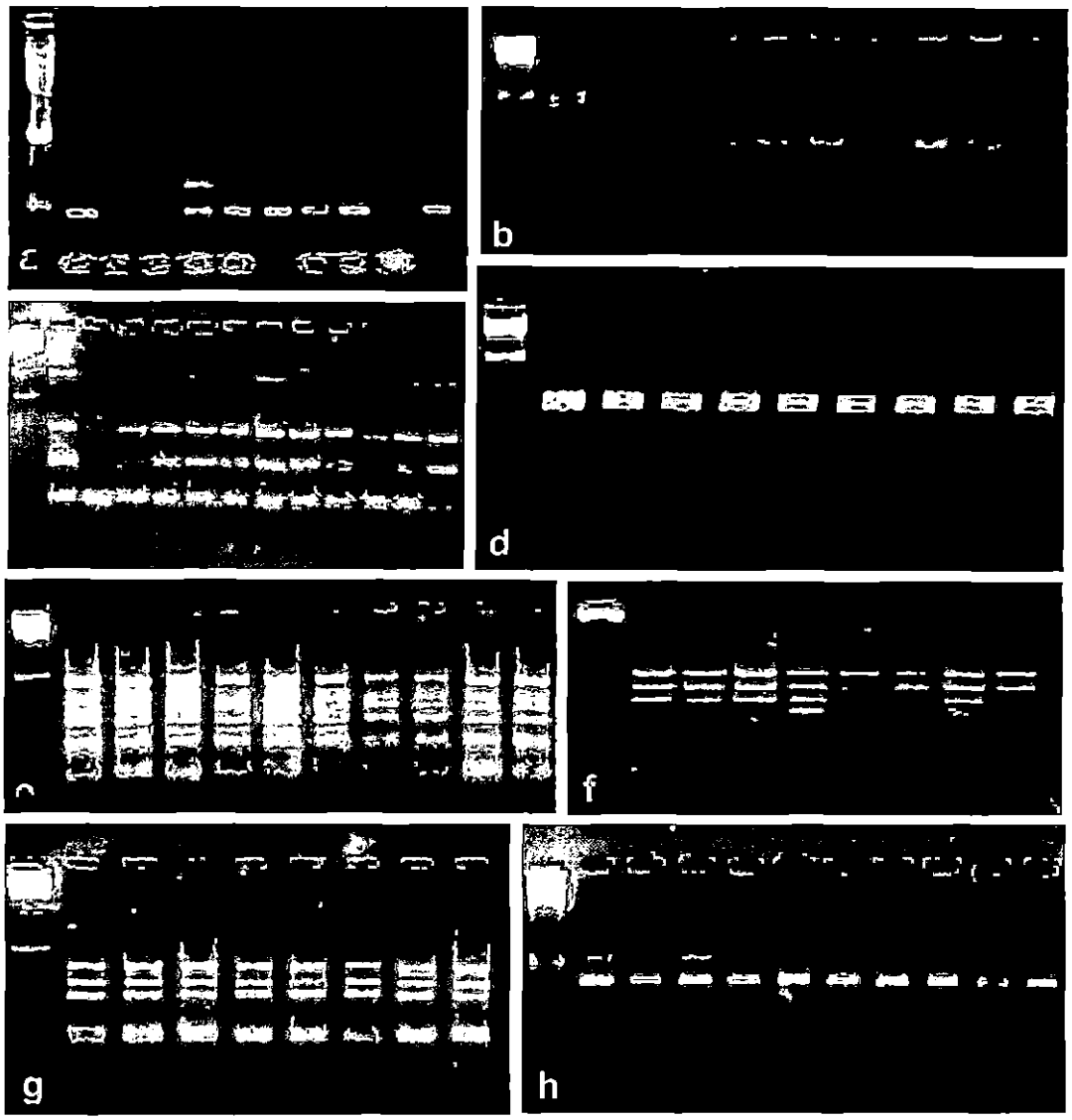

Plate VIII

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.

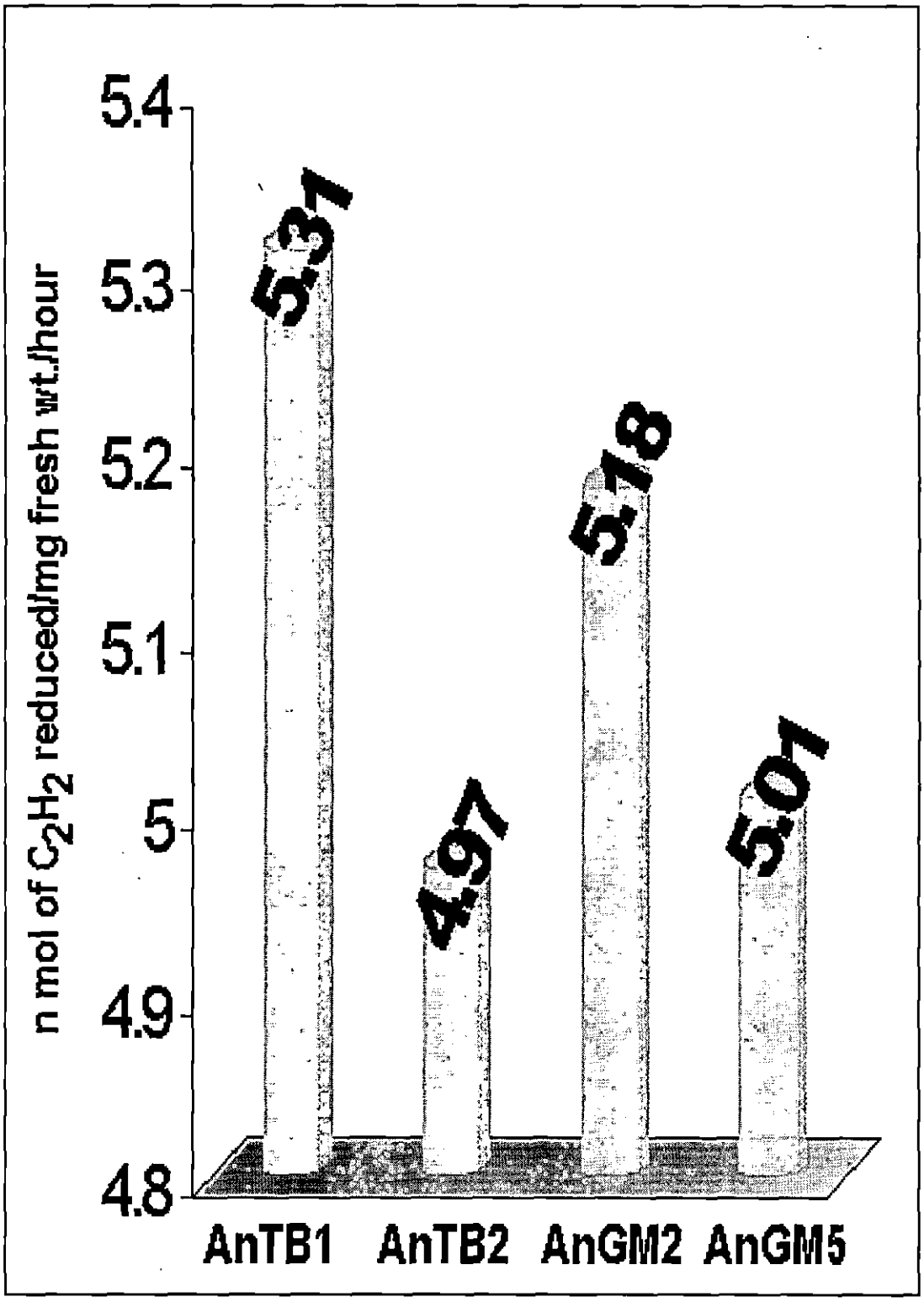

Figure 4.1: Graph showing Nitrogenase activity of *Frankia* isolates from Darjeeling region.

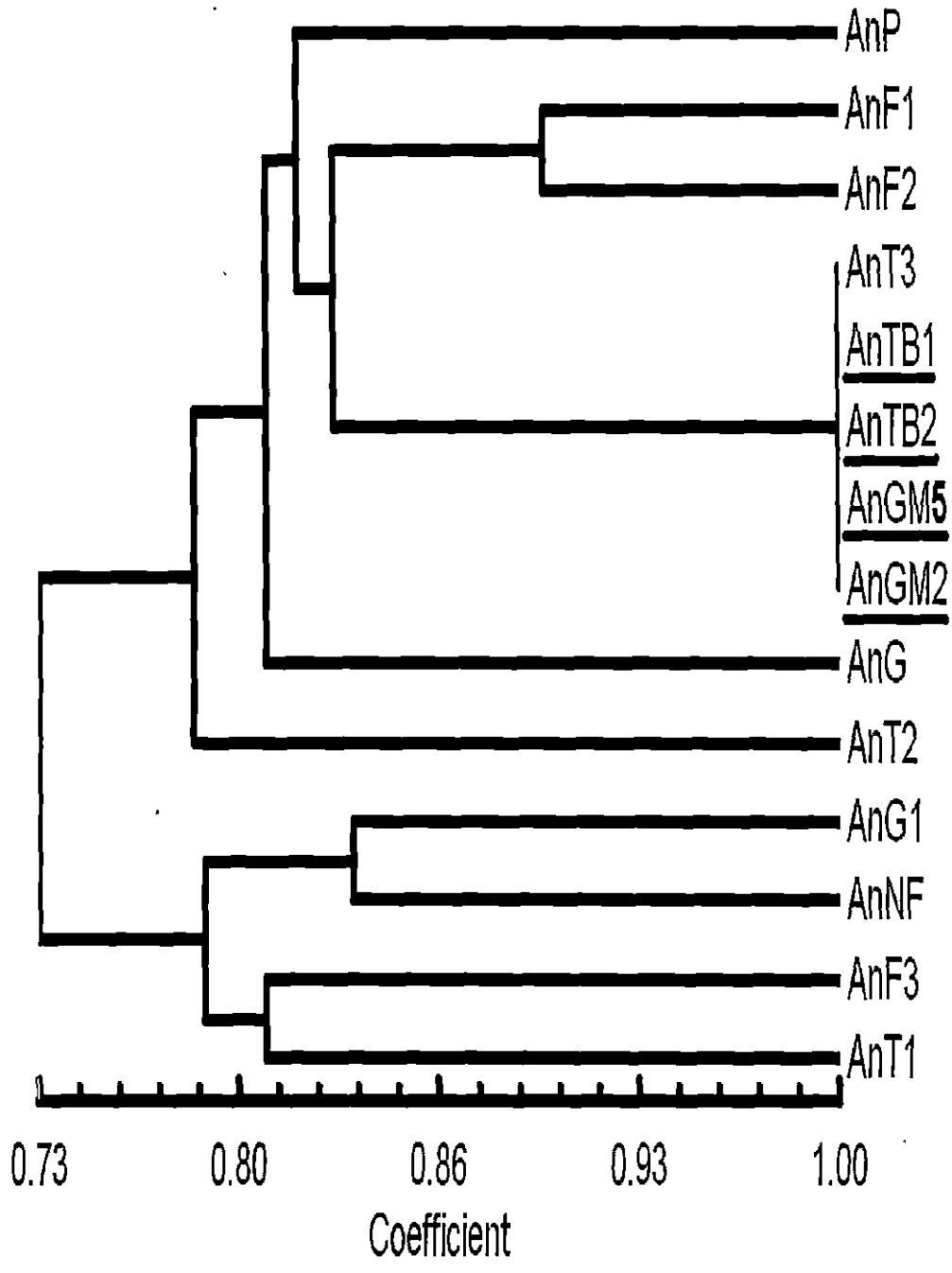

Figure 4.2: Phylogenetic tree based on PCR-RFLP pattern of 16S rRNA and *nif* HD IGS region analysed with UPGMA clustering of the matrix using NTSYSpc (Rohlf, 1994) for cultures and nodules of Darjeeling region.

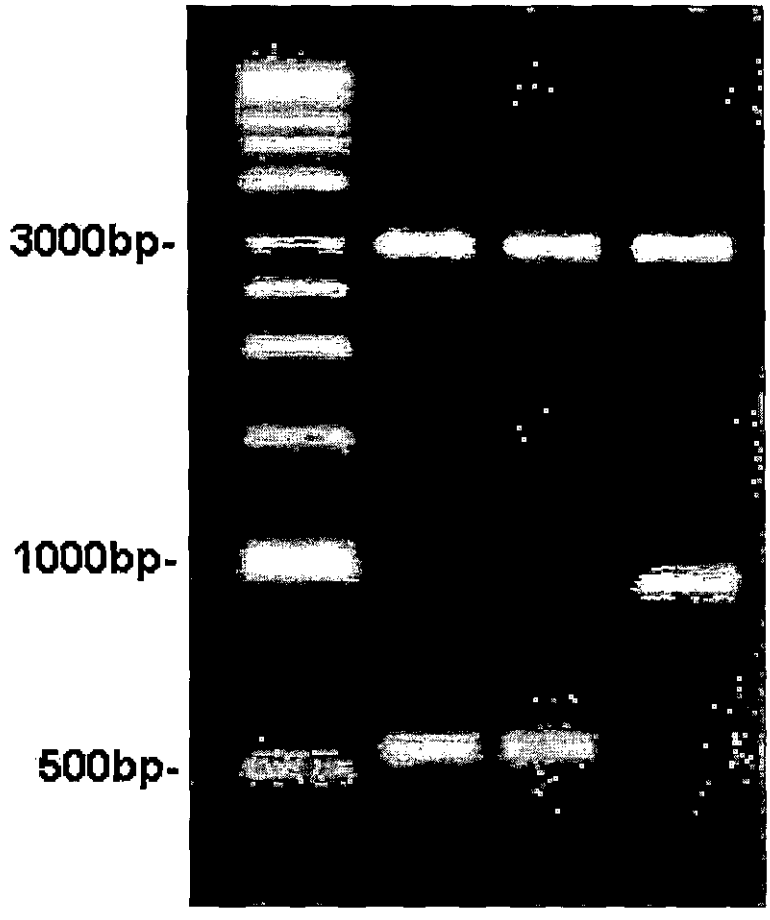

Figure 4.3: Agarose gel showing T-vector. PCR amplified products cloned in T-vector and digested with various restriction enzymes to verify the insert. Lane 1, molecular wt. ladder (Sigma Cat# D3937); Lane 2&3, distal part of 16S rRNA gene (521bp); Lane 4, nif H-D IGS region (~900bp).

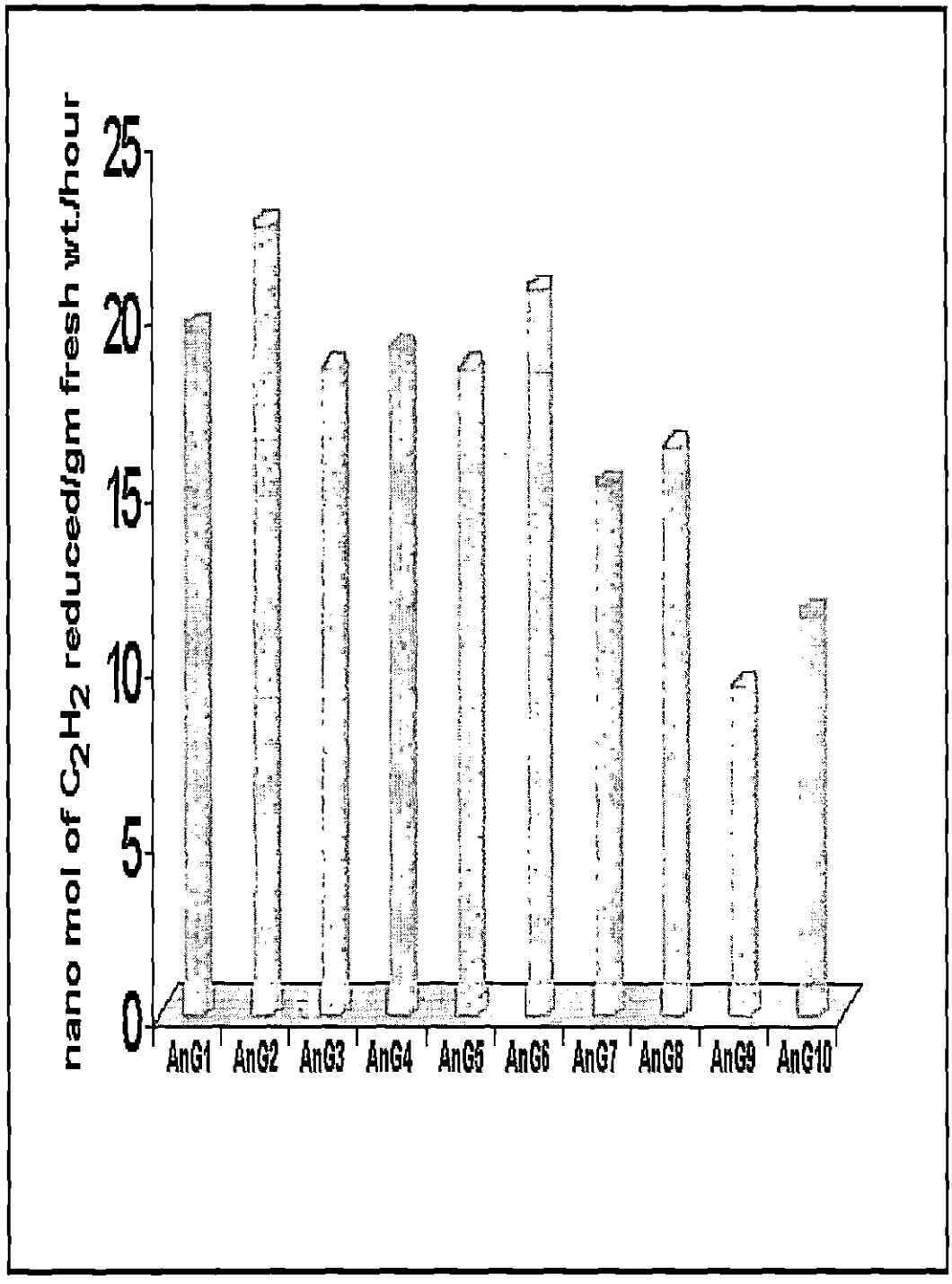

Figure 4.4: Graph showing nitrogenase activity of *Frankia* in nodules collected from different trees of *Alnus nepalensis* found in Tadong, Gangtok

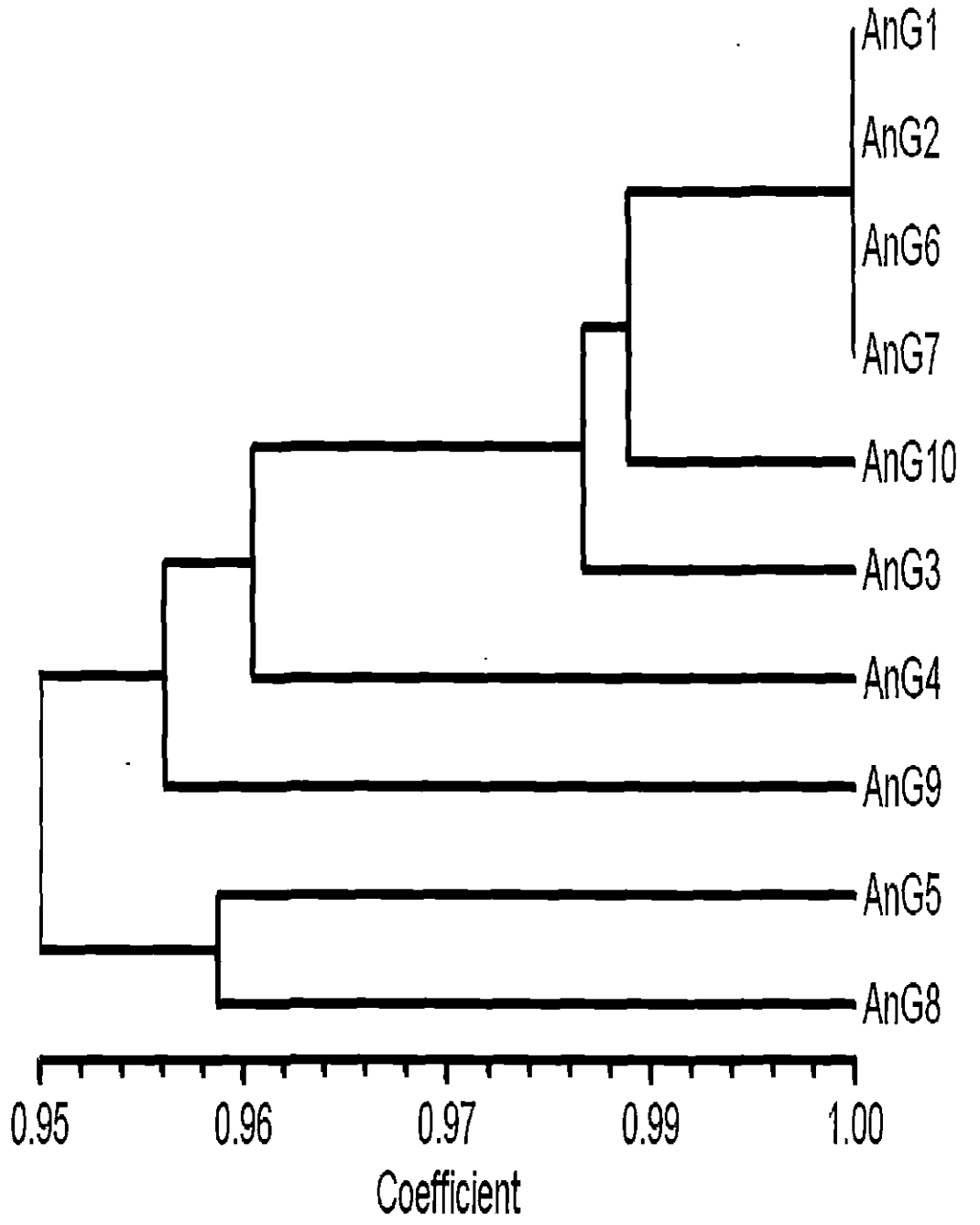

Figure 4.5: Neighbour joining consensus tree for aligned sequences of the distal part of 16S rRNA region of *Frankia* based on the PCR-RFLP pattern and analyzed with UPGMA clustering of the matrix using NTSYSpc (Rohlf, 1994) as seen in nodules collected from different trees of *Alnus nepalensis* found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