Study on the Genetic Diversity of *Frankia* in Nodule Samples of *Alnus nepalensis* Collected from Three High Altitude Sites in Sikkim

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ABSTRACT

Nodule samples from individual trees of *Alnus nepalensis* were collected from three different sites of high altitude from east and north Sikkim, India. Total genomic DNA was extracted separately from individual nodule lobes, which was then directly used for amplification. Internally transcribed spacer (ITS) region of the *rrn* operon were amplified by the polymerase chain reaction (PCR) using two primers specific for the distal part of 16S rRNA, ITS and proximal part of 23S rRNA genes of *Frankia*. Amplified products were run together to get the Amplicon Length Polymorphism (ALP).The PCR products were then digested with restriction enzyme *Rsa*1 for obtaining restriction patterns. Sizes of amplicons and distances between the RFLP patterns from these samples were found to be noticeably different, indicating presence of genetic diversity between *Frankia* populations from different sites at the same altitude.

Key words: *Genetic diversity, Frankia, Alnus nepalensis, Polymerase chain Reaction, Restriction Fragment Length Profiles, Phylogenetic Study, Amplicon Restriction Pattern.*

Introduction

The actinomycete *Frankia* is of biological interests for several reasons including its wide distribution, its ability to fix nitrogen, specialized cells for nitrogen fixation and to nodulate plants of about 24 genera (Lavire and Cournoyer, 2003). The relationship between *Frankia* and actinorhizal plants contributes greatly to global nitrogen cycle and plays a very significant role in biological

nitrogen fixation. The association is host specific, and the effectivity of strains within a plant-infectivity group can vary. As compared to the super specialized host range of *Rhizobium* confined only to the leguminous plants, *Frankia* is more versatile and dynamic organism having a wider host range of eight families and over two hundred species.

Alnus is the only genus in Betulaceae that can associate with *Frankia* to form nitrogen fixing actinorhizal root nodules. N_2-fixation by *Frankia*-actinorhizal plants is often central to the dynamics of several ecosystems. Actinorhizal plants involved in this symbiosis are important pioneer species in nitrogen poor soils or disturbed environments (Benson *et al.*, 1984). These plants play a major role in increasing the fertility of nitrogen depleted soils generated due to landslides, flooding, fire, glacial activity, volcanic eruptions, etc. Apart from these, they are also economically important as timber, fuel, medicine, wind breaks, sand dune stabilizers and in agroforestry applications. The high nitrogen content in the leaf litter increases soil fertility and can pave the way for the diversification of species within the ecosystem. The efficiency of any symbiotic partnership depends on the functional capacity of the association as well as on the environmental factors. Therefore, all types of factors should be taken into account before using *Frankia* as a biofertilizer.

Though the actinorhizal association is analogous to the much better studied *Rhizobium*-legume symbiosis, very little is known about *Frankia*, especially its ecology and genetics. Thus there is a need for obtaining basic information about the population sizes and diversity of *Frankia*. It may be noted that the first successful isolation of *Frankia* was reported only in 1978. Strain CpI1 was obtained from *Comptonia peregrina* by Callaham *et al.* (1978).

Frankia strains have shown tremendous diversity at molecular level. Therefore, a superior and efficient host microbe relationship could positively affect nitrogen-fixing capacity of the *Frankia* strain (Chauhan, 2000). And for establishing this superior and efficient relationship, most infective, effective and competitive *Frankia* strains have to be selected, which in turn require investigations on diversity existing within the species. However, only limited research has been conducted, and only a few genotypes of *Frankia* have been identified.

As a direct player in the process of nitrogen reduction, *Frankia* has an important effect on this symbiotic process. Keeping all other factors constant, it is obvious that an efficient strain would produce more nitrogen as compared to others.

Host genotype also has a dominant influence on the N_2-fixing rates of nodules. Thus nitrogen-fixing efficiency can be improved by screening for the best host and then the ideal *Frankia* strain associated with it (Verghese and Misra, 2000). Research on *Frankia*-actinorhizal symbiosis has been largely benefited from advances in molecular biology. PCR-RFLP based techniques have revealed tremendous diversity among *Frankia* strains (Nazaret *et al.*, 1991; Varghese, 2000; Verghese and Misra, 2000). Restriction fragment length profiles obtained by the digestion of genomic DNA and PCR amplified products have helped characterise a number of organisms (Jamann *et al.*, 1993; Laguerre *et al.*, 1996; Verghese and Misra, 2000). Identifying *Frankia* strains is important for several reasons. A rapid method for grouping new isolates would simplify any testing program aimed at selecting the most effective type of *Frankia* strain by narrowing down the number of strains to be tested. In addition, a method for fingerprinting strains would be useful for understanding the competitiveness and ecology of *Frankia* in the field (Benson *et al.*, 1984).

Materials and Methods

In the present study, a PCR-RFLP (also called as Cleaved Amplified Polymorphic Sequence-CAPS), based technique for *rrn* operon was used for discriminating between different *Frankia* strains. The ribosomal DNA is well-characterized ubiquitous molecular tool for estimating evolutionary relationship of organisms (Ochman and Wilson, 1987; Woese, 1987). The usual order of the structure of bacterial *rrn* operon is 16S-spacer-23S-spacer-5S with few exceptions. These ribosomal RNA genes are evolutionarily homologous and functionally equivalent in all organisms. Any changes in their sequence are slow which allow the estimation of evolutionary relationships between even distantly related organisms. The Internal Transcribed Spacer (ITS) region in prokaryotic rRNA genes are situated between 16S and 23S and between 23S and 5S genes. These are varied in length and are assumed to have been subjected to less selection pressure. Therefore, they should

have accumulated more random mutations than the coding regions. Thus, it forms an ideal region for the discriminative studies (Varghese, 2000).

Study area

Study was conducted in Sikkim, which is situated in north-east India, surrounded by Nepal in the west, China in the north, West Bengal in the south and both Bhutan and China in the east. Sikkim is part of a hot spot of biodiversity and is situated approximately between 88°E–89°E longitudes, 27°N–28°N latitudes and at a range of 300–8545 m above the mean sea level. Study was conducted only in north and east Sikkim districts.

Description of sites: Three different sites were selected at a high altitude range above 2000 m. This height was considered as "high" because *Alnus* trees are generally not found beyond 2500 m. Two sites were situated in east Sikkim, one on the way to Hanumantok, Gangtok and the other one inside the Himalayan Zoological Park, Bulbulay, Gangtok. The third site was situated in north Sikkim district, on the way from Chungthang to Lachung.

High Altitude, Site 1 (On the way to Hanumantok, Gangtok), 2100 m — Samples were collected mainly from trees on the roadside slopes. The average age of the trees in this site was five to eight years. Solitary *Alnus* trees were found growing among other trees. Tree no. 10 was located near one small streamlet. The soil was porous, loose, moist to dry and full of raw organic matter. In some places, the soil looked sandy and grayish brown.

High Altitude, Site 2 (Himalayan Zoological Park, Bulbulay, Gangtok), 2200 m — Samples were collected from the zoological park. The average age of the trees was between seven to ten years. The soil was basically loose in texture, sandy in most places and grayish brown in colour.

High Altitude, Site 3 (Between Chungthang and Maltin), 2040 to 2067 m — Samples were collected from young to middle aged trees from a site located on the roadside, approximately one kilometer before Maltin and eight kilometer from Chungthang. Age of the trees varied from 3 to 15 years. Soil was light brown in color, sandy mixed with rocks and loose.

Sample collection: Nodule samples were collected from 30 individual trees, ten from each site. The nodule samples were collected in plastic bags, properly labeled and brought back to the laboratory. On arrival to the lab, nodule clusters from each tree were washed thoroughly under running tap water, air dried and then stored at –20°C for further use.

DNA isolation: Extraction of total genomic DNA from individual nodule lobes was carried out following the methodology given by Rouvier *et al.* (1996) with minor modifications. A single nodule lobe was first sterilized in 30% hydrogen peroxide (H_2O_2) for one or two minutes. After that it was repeatedly washed in autoclaved distilled water for three to four times. Then the outer epidermis was peeled off with a sterilized needle and the nodule lobe was transferred to a 1.5 ml microcentrifuge tube. The lobe was then crushed properly and about 350 ml of warm DNA extraction buffer along with 10 ml of 20% SLS (sodium lauryl sulphate) were added to it. The tube was then kept in a waterbath for one hour at 65°C. After the incubation the tube was centrifuged at 8000 rpm for about 7 minutes at room temperature. The supernatant was then transferred to another fresh tube and an equal volume of chloroform-isoamyl alcohol (24:1) was added to it. The tube was centrifuged again at 13,000 rpm for 15 minutes at room temperature. The upper aqueous layer was transferred to another fresh tube and 2.5 volumes of ice cold ethanol was added. It was kept overnight at –20°C for precipitation. Next day the tube was centrifuged at 13,000 rpm for 30 minutes at 4°C. Ethanol was discarded and the DNA precipitate was washed twice with 350 ml of 70% alcohol. Later the DNA precipitate was vacuum dried, dissolved in ultrapure water and stored at –20°C for future use.

Amplification of genomic DNA by Polymerase Chain Reaction (PCR): Samples containing good amount of isolated DNA were subjected to PCR amplification. Each reaction mixture contained 2.5 µl of 10 µM primer, 2.5 µl of 10X PCR buffer, 2.5 µl of 25 µM $MgCl_2$, 10 µl of 5mM dNTP mix, 0.75 µl of *Taq* polymerase (3u/µl), 1 µl of template DNA and ultrapure water to make the total volume to 25 µl of PCR mix per tube. Amplification reaction was carried out for 35 cycles. Each cycle comprised of 1 min denaturation at 94°C, 1 min annealing at 49°C and 1 min of elongation at 72°C. A hot start was given for five minutes at 94°C and at the end of the

35 cycles an additional 7 minute extension time at 72°C was added to allow the partially amplified DNA to fully amplify.

Amplification of *rrn* ITS was done using forward primer FGPS 989 (5'GGGGTCCGTAAGGGTC3', Bosco *et al.*, 1992) and reverse primer FGPL 2054' (5'CCGGGTTTCCCCATTCGG3', Simonet *et al.*, 1991).

Quantification of DNA by agarose gel electrophoresis: Both isolated and amplified DNA samples were run in a 0.8% agarose gel at 70 volts for one and half hours to check for the presence of DNA. The gel was then stained in ethidium bromide for about fifteen minutes and then scanned and photographed using BIO-RAD GelDoc1000 and Multi-Analyst software (version 1.1).

Amplicon Length Polymorphism (ALP): Amplicons of different samples were subjected to gel electrophoresis together to detect the ALP. The ALP was photographed using BioRad GelDoc1000.

Restriction digestion of amplified products (RFLP): Restriction digestion was done for all thirty samples from high altitude sites. Suitable restriction enzyme, *Rsa*1 was selected for this purpose and the samples were digested overnight at 37°C. The restriction digestion mixture was prepared in a 0.5 µl tube in which 8 µl of the amplicon was digested with 5 units of the restriction enzyme in the appropriate buffer solution of 2 µl and the remaining volume was made up with ultra pure distilled water to make a final volume of 20 µl. After digestion was over, the digested samples were run in ethidium bromide stained 4% agarose gel at 50 volt for 5–6 hrs. The gel was then scanned and photographed using BioRad GelDoc 1000 and Multi Analyst software.

Results

DNA Isolation: Isolation of DNA from all the samples was successful, as visualized on the agarose gels. The amount of DNA was estimated visually, and appropriate dilution factor was determined for use in subsequent DNA amplification studies.

Polymerase Chain Reaction (PCR): PCR yielded bands of expected sizes (Fig. 1) under the conditions described above. These amplicons were used for ALP and ARP studies as below.

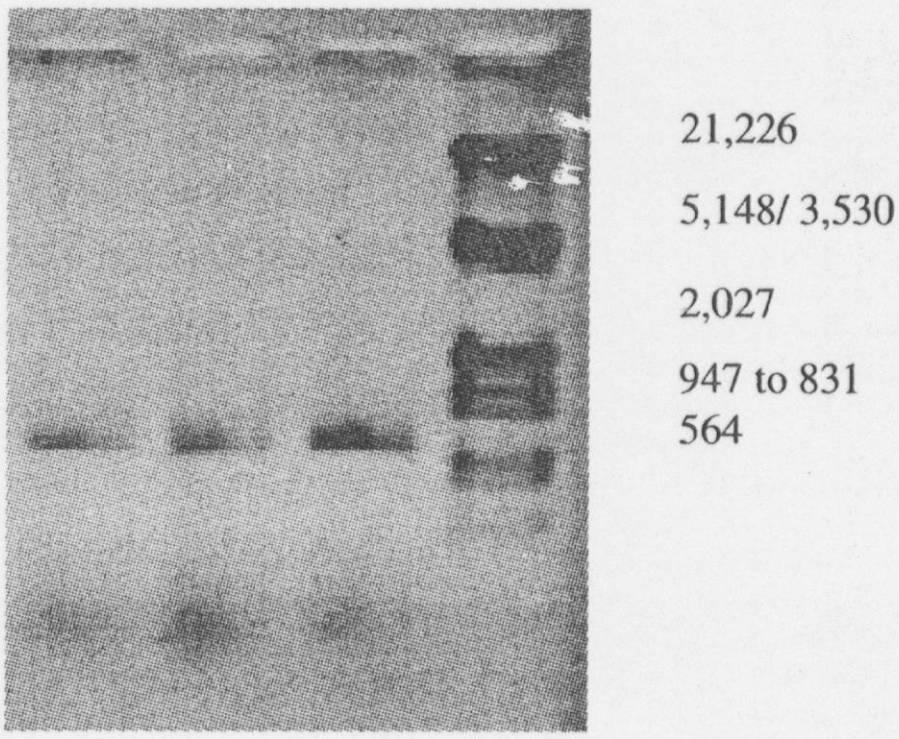


Fig. 1. Amplification of 16S-ITS-23S rRNA region. (L to R Lane 1–3 amplified DNA, L 4 DNA marker).

Amplicon Length Polymorphism (ALP): There was a distinct size variation with respect to the target region amplified as above in terms of the base pairs. The amplicons ranged between approximately 1100 to 1500 bp. (Fig. 2).

Restriction Fragment Length Profile (RFLP): Restriction patterns with *Rsa*1 revealed considerable variation. The variation pertained to both the size of bands as well as the number of bands for different samples (Fig. 3). Based on this, at least six different patterns were identified.

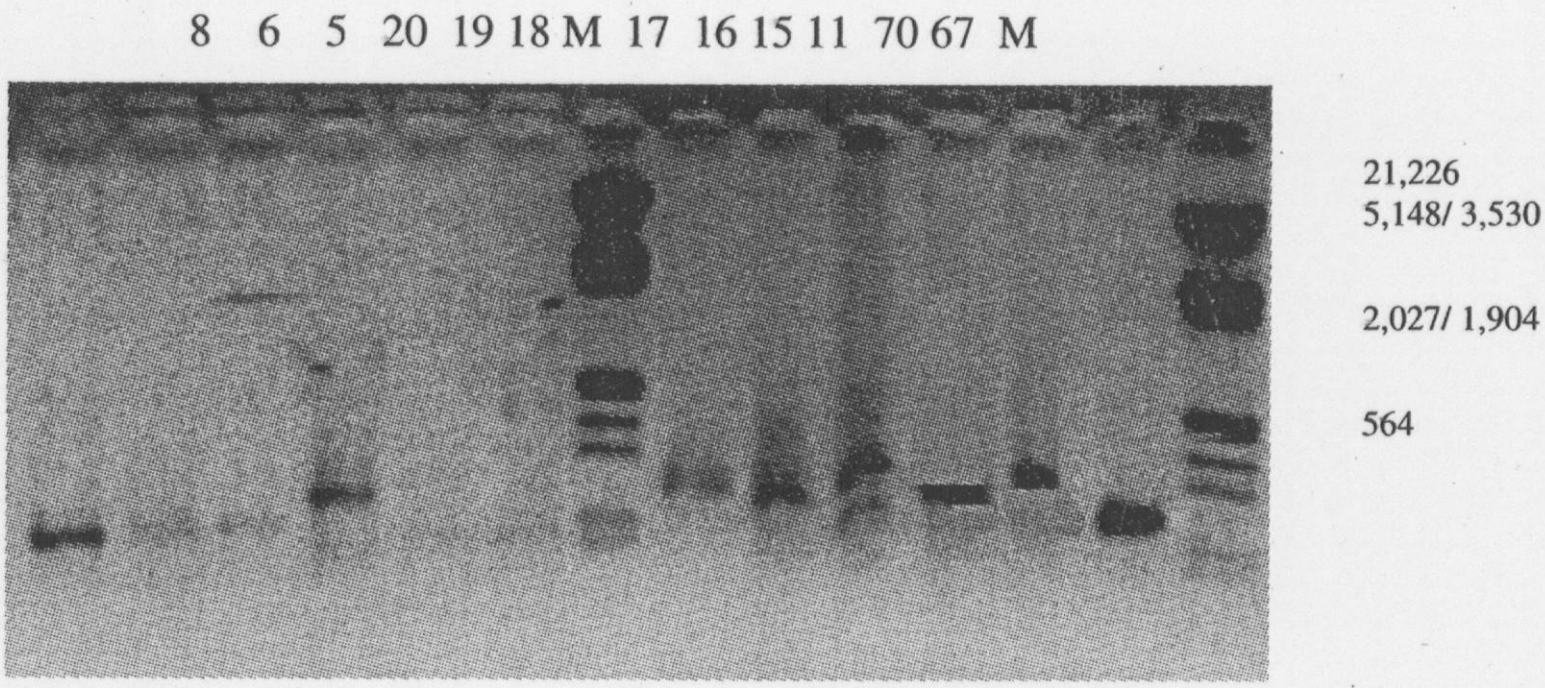
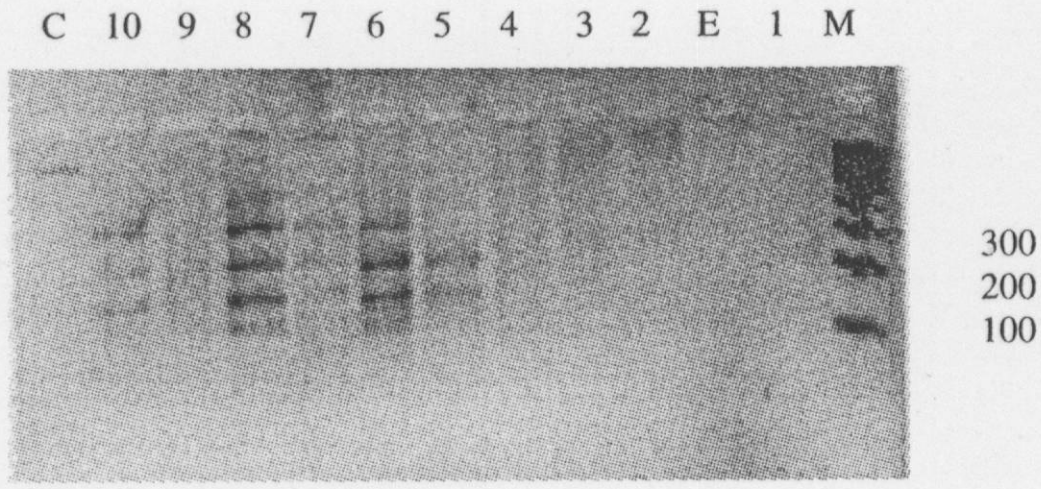
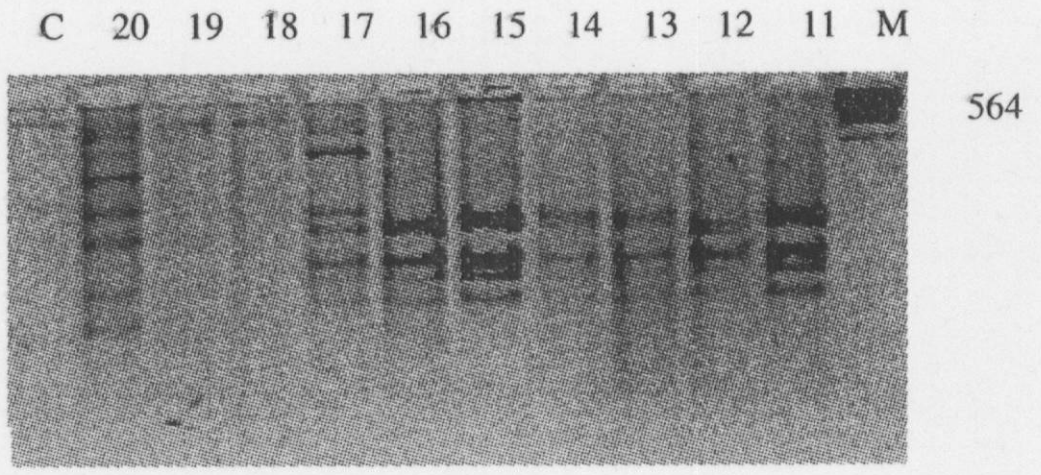


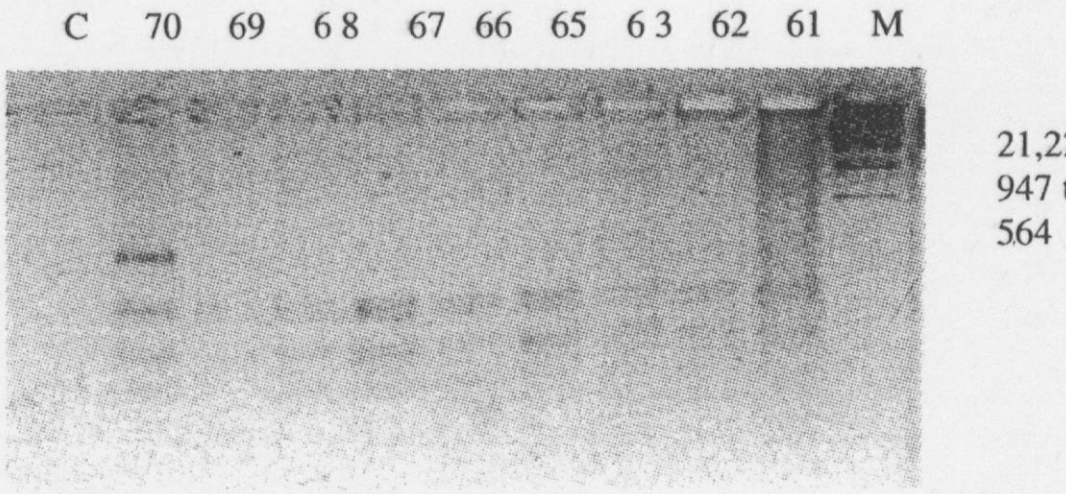
Fig. 2. Gel photograph showing ALP. Legends at the top indicate sample numbers. M = DNA marker.



Site 1. Gangtok, East Sikkim



Site 2. Gangtok, East Sikkim



Site 3. North Sikkim

Fig. 3. Gel Photographs showing different RFLP patterns obtained (M–Molecular marker, E–empty lane, C–Undigested Control).

Discussion

Amplified Fragment Length Profile (Amplicon Length Profile) — ALP: Agarose gel electrophoresis of amplicons of different samples revealed a variation in their patterns and sizes. For examples, two bands were obtained in sample no. 15 of higher altitude, site 2. This indicated a size variation in the intervening region for the primer pair's annealing sites. This is not entirely unexpected as some deletions/additions in the ITS region may not have any adverse impact on the organisms and may therefore get fixed. Further, *Frankia* is known to have two operons for *rrn* region. If an

event of addition/deletion in one of the operons occurs, amplicons with different sizes will be generated. Therefore, ALP could detect existence of variation in populations of *Frankia* at different sites.

Restriction Fragment Length Profile (Amplicon Restriction Profile-ARP): Restriction digestion of above amplicons with *Rsa*1 revealed the presence of at least six different patterns. Some of these patterns were present in all the different sites. However, some of these patterns appeared to be confined to a given site, for example, the pattern obtained for sample no. 5, higher altitude, site 1, was unique. The pattern represented by sample nos. 21 to 29 is also confined to this particular site. Apparently, the distribution of strains was site-specific.

Therefore, our approach demonstrated the presence of variability at molecular level. It also revealed a site dependent distribution of *Frankia* genotypes in Sikkim region.

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