Polymorphism in *Alnus* based *Frankia* of Darjeeling

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DNA samples extracted from the root nodules of *Alnus nepalensis*, collected from 10 different locations of Darjeeling hills, were used to assess the genetic diversity of *Frankia*. The DNA samples from the nodules of naturally growing plants were used as templates in PCR, targeting different genomic regions of *Frankia*, namely distal, middle and proximal parts of 16S rRNA gene and nifH-D IGS region with locus specific primers. The PCR products were digested with a number of frequent (4-base) cutter restriction endonucleases. Bands were scored as present (1) or absent (0) and the clustering was done using NTSYSpc. Distinct polymorphism was found among the nodules collected from different parts of the region and those of same geographic area. These results demonstrate that genetic diversity is indeed present among the naturally occurring *Frankia* of Darjeeling, India.

**Keywords:** Actinorhizal plants, *Frankia*, NTSYSpc, PCR-RFLP, 16S rRNA.

A diverse group of microorganisms shows an ability to fix molecular nitrogen either in free state or in symbiotic association with plants. Among the symbiotic nitrogen fixers, other than *Rhizobium*, an actinomycete called *Frankia* is most important. Actually the amount of nitrogen that *Frankia* fixes on a global basis is more than that of *Rhizobium*.

Unlike *Rhizobium*, *Frankia* makes symbiotic association with a large number of woody dicotyledonous genera, belonging to 8 different families. These plants are called actinorhizal plants. Among the actinorhizal plants found in India, *Alnus nepalensis* D. Don (Alder) of Betulaceae is the most important one.

*Alnus* grows in marginally fertile soils and often serves as a pioneer species early in the successional plant community development. As a nitrogen-fixing species it is suitable for soil improvement and rehabilitation of degraded lands. In hilly areas prone to land sliding, seeds of *Alnus* have been broadcast to stabilize landslides. In Burma, *Alnus* has been used with success to reforest abandoned Taungya areas. In agroforestry systems it can be planted at borders with many crops. The wood of the plant is a source of firewood and charcoal. In India, *Alnus* is mostly found in the cold temperature areas of Northeast including the hills of Darjeeling where they are especially important because the indigenous legumes are mostly absent or rare (data not shown).

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**Materials and Methods**

The root nodules of *Alnus nepalensis* D. Don (Fig. 1) were collected from healthy and young trees growing locally in Darjeeling hills. The nodules were found at a depth of 7-30 cm and carefully excavated along with a small portion of the attached root system.

Only young nodules of light brown colour were collected because they show active growth.

Various factors like size of nodules and presence of plant phenolics make the isolation of DNA from alder...
(A. nepalensis) root nodules a tedious process. We adopted the method of Rouviere et al.\textsuperscript{5} with minor modifications by incorporating RNase treatment. RNase was added at a rate of 50 \( \mu \text{g} \/ \text{ml} \) and incubated for 1 hr at 37\(^\circ\)C in a dry bath (Genei, India).

Yield and purity of a DNA were checked with the help of UV-Vis Spectrophotometer (Thermo-Spectronic UV1) at OD\(_{260}\). Purity of a DNA sample was calculated from ratio of OD\(_{260}/OD_{280}\).

DNA amplification was performed with GeneAmp 2400 thermal cycler (Perkin-Elmer, USA) under the conditions – (1) initial denaturation at 94\(^\circ\)C for 5 min and 35 cycles of denaturation (1 min at 94\(^\circ\)C); and (2) annealing (1 min) at 55\(^\circ\)C, extension (1 min at 72\(^\circ\)C), and final extension for 7 min at 72\(^\circ\)C. The reaction mixture (50\( \mu \text{l} \)) contained 5\( \mu \text{l} \) of diluted DNA (~30 ng), 1XPCR buffer (pH 8.3), 1.5 mM of MgCl\(_2\), 0.5\( \mu \text{M} \) of each of primers and 3U of Taq DNA polymerase (all from Sigma-Aldrich, USA). Annealing temperature depends upon the size and nature of the primers and was calculated with the following formula—Annealing temp. (\(^\circ\)C)=\([2(A+T)+4(G+C)]\)/5. When two primers (Forward and Reverse) had different annealing temperatures, the lowest one was used. Primers shown in the Table 1 were used for amplifying different parts of \textit{Frankia} genome. Amplification of DNA was checked by agarose gel electrophoresis (0.8\% w/v) with 5 \( \mu \text{l} \) of PCR product and the gel was stained with ethidium bromide.

PCR products were digested for 1 hr at the optimal conditions as mentioned in Table 2. The reactions were performed in a cooling dry-bath (Bangalore Genei, India). Restriction fragments were separated by electrophoresis on 3\% (w/v) molecular biology grade high-resolution agarose (Sigma-Aldrich, USA) gel containing ethidium bromide (0.55 \( \mu \text{g} \/ \text{ml} \)) and documented with Kodak Digital Science gel documentation system on a UV-Transilluminator (Gibco-BRL). The PCR RFLP data were scored as present (1) or absent (0) using the SIMQUAL program and DICE coefficients. A matrix was computed and phenograms were developed by a UPGMA clustering of the matrix NTSYS-pc\textsuperscript{10}.

**Results and Discussion**

Sufficient quantity of DNA was extracted from a single nodule lobe for PCR amplification using the method described by Rouvier et al.\textsuperscript{5} with modifications made by incorporating RNase treatment followed by one more purification step.

When isolated DNA was subjected to PCR amplification, most of the samples gave positive results. Different primers successfully amplified distal, middle and proximal parts of 16S rRNA gene and intergenic spacer region (IGS) of \textit{nif} H-D of \textit{Frankia}.

### Table 1—Primers used to amplify different regions of \textit{Frankia} genome

<table>
<thead>
<tr>
<th>Target Regions</th>
<th>Primer sequences*</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{nif} HD-IGS region</td>
<td>FGP-750 5‘GAGACGATCCGGACCGA3’</td>
<td>Simonet et al.\textsuperscript{15}</td>
</tr>
<tr>
<td></td>
<td>FGP-286 5‘TTCATCGGACGATGCTG3’</td>
<td>- do -</td>
</tr>
<tr>
<td>16S rRNA (distal)</td>
<td>FGP-989ac 5‘GGGGTGCGTAAGGGTC3’</td>
<td>Bosco et al.\textsuperscript{11}</td>
</tr>
<tr>
<td></td>
<td>FGP-1490 5‘AAGGAGGGGATCCAGCCCGA3’</td>
<td>Norman et al.\textsuperscript{14}</td>
</tr>
<tr>
<td>-do- (middle)</td>
<td>FGP-485 5‘CACAGCAGCGCGTAA3’</td>
<td>- do -</td>
</tr>
<tr>
<td></td>
<td>FGP-910 5‘AGCGCTGGGCGTACTCCC3’</td>
<td>- do -</td>
</tr>
<tr>
<td>-do- (proximal)</td>
<td>FGP-6 5‘TGGAAAGCTTGGTGGA3’</td>
<td>- do -</td>
</tr>
<tr>
<td></td>
<td>FGP-505 5‘GTGATTAATCCCGGCTG3’</td>
<td>- do -</td>
</tr>
</tbody>
</table>

*Primer numbers for rRNA genes are as per \textit{E. coli} numbering\textsuperscript{12} and for \textit{nif} genes are as per \textit{K. pneumoniae} numbering\textsuperscript{13}.

### Table 2—List of restriction enzymes used in the present study

<table>
<thead>
<tr>
<th>RE (with Cat# of Genei, India)*</th>
<th>Sequence</th>
<th>Reaction volume (( \mu \text{l} ))</th>
<th>Reaction temp. ((^\circ)C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alul (MBE-17S)</td>
<td>AG_CT</td>
<td>20</td>
<td>37</td>
</tr>
<tr>
<td>TaqI (MBE-7S)</td>
<td>T_CGA</td>
<td>20</td>
<td>65</td>
</tr>
<tr>
<td>HinfI (MBE-21S)</td>
<td>G_ANTC</td>
<td>20</td>
<td>37</td>
</tr>
<tr>
<td>HaeIII (MBE-10S)</td>
<td>G_CC</td>
<td>20</td>
<td>37</td>
</tr>
<tr>
<td>Hhal (MBE-14S)</td>
<td>CC_GC</td>
<td>20</td>
<td>37</td>
</tr>
<tr>
<td>MboI (MBE-27S)</td>
<td>G_ATC</td>
<td>20</td>
<td>37</td>
</tr>
<tr>
<td>MspI (MBE-31S)</td>
<td>C_CGG</td>
<td>20</td>
<td>37</td>
</tr>
<tr>
<td>HindIII (MBE-6S)</td>
<td>A_AGCTT</td>
<td>20</td>
<td>37</td>
</tr>
<tr>
<td>EcoRI (MBE-35)</td>
<td>G_ATTTC</td>
<td>20</td>
<td>37</td>
</tr>
</tbody>
</table>

RE—Restriction enzyme
Amount of each restriction enzyme used was 5 units

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Amplification of distal region of 16S rRNA gene was done using primer FGPS-989ac and FGPS-1490'. Primer FGPS-989ac, originally developed by Bosco et al., 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 was typical as reported earlier. It confirmed the presence of Frankia in the nodules. The DNA samples from all the 50 nodules collected from 10 plants of various locations of study were amplified (Fig. 2).

The proximal part of 16S rRNA gene was amplified using primers FGPS6 and FGPS505', while the middle part of 16S rRNA gene was amplified using primers FGPS485 and FGPS910'. In both the cases expected bands of 540 and 415 bp sizes were observed. Despite of several trials, in a few cases DNA could not be amplified with primers FGPS6 and FGPS505'. This indicated that there were major nucleotide substitutions present in this particular region of DNA.

In case of middle part of the 16S rRNA gene in all the cases amplification took place. Initially at 55°C annealing temperature more than one bands in place of the expected one, were observed. As per Verghese and Misra this may be due to the amplification of plant DNA along with Frankia DNA. By raising annealing temperature by 2°C, a single band of the size of 415 bp was obtained. There was a possibility that this amplification product could be either of Frankia origin or plant origin of chloroplast 16S rRNA gene. It was further tested by digesting with EcoR I, since this region of plant chloroplast DNA does not have an EcoR I site. Our products were digested well with EcoR I and produced 155, 135 and 125 bp fragments.

Besides this, the \textit{nif} H-D intergenic spacer (IGS) region was amplified with primers FGPH-750 and FGPD-826'. However, unlike Verghese and Misra

![Fig. 3—Phylogenetic tree based on PCR-RFLP pattern analyzed with UPGMA clustering of the matrix using NTSYSpc10 (Rohlf).](image)

![Fig. 2—(a) and (b) Agarose gel electrophoresis of PCR products of distal part of 16S rRNA gene and \textit{nif} H-D IGS region, respectively. (c) and (d) Restriction patterns of the distal part of 16S rRNA gene digested with \textit{Hinfl} and \textit{nif} H-D IGS region digested with TaqI, respectively.](image)
who have reported multiple bands, we observed single band of around 900 bp long. All PCR products discussed above were subjected to restriction digestion with various restriction enzymes (Table 2). High level of polymorphism was found in distal and proximal parts of 16S rRNA genes and nif H-D IGS region (Fig. 2). These data were further analyzed with the help of UPGMA software NTSYSpc\(^{10}\) (Fig. 3).

Analysis of variance of image of Frankia 16SrRNA gene and nif H-D IGS region showed that significant differences existed among nodules collected from different parts of Darjeeling hills. Nodules collected from two sites from Fatak (AnF1 and AnF2) were identical, whereas nodules collected from other sites of Fatak were distantly related to the above two nodules and close to the nodules of one site of Thurbo. On the other hand, AnT3 was close to AnF1 and AnF2. Nodules from Ghoom region i.e. AnG and AnG1 were not closely related. We tried to correlate these results with the altitude i.e. whether the genetically close Frankia strains belonged to more or less same elevation. It was found that there was no correlation. For example, nodules found in Ghoom at an altitude of 7407 ft. are close to the nodules found in New Fatak region at an altitude of around 3000 ft., whereas nodules from different parts of Fatak located at same altitude were not so close (Fig. 3).

Therefore from the present study, it was concluded that PCR-RFLP could detect polymorphism among different Frankia strains and considerable variation existed among the Frankia of Darjeeling hills. The distributions of Frankia strains were not dependent on the altitude of the sites.

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References