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ORIGINAL PAPER

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# Development of molecular markers for screening of *Alnus nepalensis* (D. Don) genotypes for the nitrogenase activity of actinorhizal root nodules

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**Abstract** *Alnus nepalensis* (D. Don), an alder species, is an actinorhizal tree found in the hilly regions of Eastern and Northeastern India. It is useful in the reclamation of wastelands generated by land slips, shifting agriculture and coal mining. To maximise the soil regeneration capacity of alder plantations, it would be useful to be able to select superior alder genotypes at the nursery level. Conventional methods of genotype screening are difficult to apply to open-pollinated forest trees. It would be beneficial if molecular markers could be developed for early screening. The study reported here was conducted to assess the feasibility of developing PCR-based AFLP and RFLP screening tools for the selection of superior genotypes of this valuable tree with respect to their ability to support efficient nitrogen-fixing root nodules. It was found that a multi-site strategy, including the chloroplast *rrn* operon and the nuclear rRNA genes, yielded promising results. A molecular marker for genotypes that support nodules with low nitrogenase activity was also identified.

**Keywords** *Alnus nepalensis* · PCR-RFLP markers · Nitrogenase activity

## Introduction

The *Frankia*-actinorhizal plant symbiosis is an important association capable of fixing atmospheric nitrogen. The efficiency of this symbiosis depends both on the host plant and the associated symbiont (Sougoufara et al. 1992; Verghese and Misra 2000). Both partners can influence each other's physiology and in turn influence the efficiency of nitrogen fixation. Identification of the best host genotype × *Frankia* strain combination could enhance the efficacy of this symbiosis. Attempts have already been made to develop a screening method for superior *Frankia* genotypes found in the root nodules of *Alnus nepalensis*, an alder species found in India (Ganesh et al. 1994; Varghese 2000). However, similar efforts focused on early screening of the host tree have not been reported previously.

*A. nepalensis* is an open-pollinated forest tree found in the hilly regions of Eastern and Northeastern India. It belongs to the family Betulaceae, and *Alnus* is the only genus in this family that can associate with *Frankia* to form nitrogen-fixing actinorhizal root nodules. *A. nepalensis* is used as a pioneer plant in forest regeneration and for the reclamation of nitrogen-depleted soils. The use of conventional breeding techniques to optimise the alder-*Frankia* symbiosis would be time-consuming and difficult. A reliable and efficient tool for the early classification of nursery trees could be a boon for screening alder genotypes.

The aim of the present study was to assess the feasibility of developing molecular markers for the early screening of *A. nepalensis* trees in natural populations with reference to the nitrogen-fixing ability of their root nodules. To achieve this, 100 trees were screened for nitrogenase activity, and PCR-based AFLP/RFLP profiles were constructed for each tree, in order to identify genomic markers that could be used to predict nitrogen-fixing capacity.

## Materials and methods

### Collection sites

Two forest stands at Shillong, Meghalaya, were selected for study. One site was at Upper Shillong, located at 25° 32' 52" N (latitude) and 91° 53' 10.5" E (longitude) at an altitude of approximately

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6000 ft. The other site was on the Permanent Campus of North Eastern Hill University (NEHU), Mawlai, located at 25° 62' 14.9" N (latitude) and 91° 89' 7.8" E (longitude) and at an altitude of approximately 4500 ft. The two sites are separated by a road distance of approximately 20 km. The sites were selected to ensure that a wide range of *Frankia* strains were represented in our sample.

At the Upper Shillong site, the trees used were growing in four clusters. Trees 1–15 were located along a stream, trees 16–30 were very close to the first cluster but further away from the stream, trees 31–40 were located across a small patch of grassland but near the stream, and trees 41–50 were on the same side but further away from the stream. The other prominent actinorhizal trees found in the area included *Myrica* sp. and *Elaeagnus* sp.

At the NEHU Permanent Campus, the trees were more dispersed. The only prominent cluster of alder trees comprised about twenty trees. All other alder trees sampled were sited along the various roads on the campus. None of the alder trees was near any perennial water source. Although both *Myrica* and *Elaeagnus* are found in this general area, they were not found in close proximity to the alders.

## Collection of leaves

Fifty trees were randomly selected at each of these two sites. The trees selected for the study were numbered PC 1–50 at the NEHU Permanent Campus, Mawlai and US 1–50 at Upper Shillong. Infected and unhealthy leaves were avoided and only young leaves were collected from each tree. These leaves were kept in labelled polythene bags. Leaves from ten trees were collected in a day and brought to the lab.

## Collection of nodules

Alder nodules were collected in the months of September and October. The weeds around each labelled alder tree were cleared and the soil around the tree was carefully dug to expose the roots. The roots were traced to the tree to ascertain their origin. Clusters of nodules were collected from these trees and put into appropriately labelled plastic bags and brought to the laboratory. Only fresh light-brown nodules were collected. The nodules were washed under running tap water to remove mud. They were then washed with a mild detergent and rinsed thoroughly. This was followed by surface sterilisation for 2–3 min using 30% $H_2O_2$. They were then rinsed again with sterile distilled water. The sterilised nodules were used to assess the nitrogenase activity so that any nitrogen fixer present on the nodule surface did not interfere with the observations.

## Isolation of DNA

Total genomic DNA was isolated from *A. nepalensis* leaves using a modification of the protocol of Rouvier et al. (1996) for the isolation of DNA from nodules. Young, uninfected leaves were surface sterilised using 30% $H_2O_2$ and then thoroughly rinsed with ultrapure water. Two leaves were crushed in a mortar in 1 ml of warm extraction buffer [1 M TRIS-HCl, 5 M NaCl, 0.5 M EDTA, 2% (wt/vol) cetyltrimethlyammonium bromide (CTAB) and 3% (wt/vol) polyvinyl pyrrollidone (PVPP)]. The macerate was filtered through cotton into a 1.5-ml microcentrifuge tube and centrifuged for 5 min at 8000×*g* at room temperature. The supernatant was discarded and the pellet re-suspended in 300 µl of the extraction buffer. Then 10 µl of 20% SDS was added and gently mixed, and the homogenate was incubated in a water bath at 65°C for 1 h. It was then centrifuged at 12,000×*g* for 15 min. The supernatant was transferred to a fresh tube and extracted with equal volume of chloroform:isoamyl alcohol (24:1). After centrifugation at 12,000×*g* for 15 min, the aqueous phase was transferred to a fresh tube, and 30 µl of 3 M sodium acetate (pH 5.2) and 1.2 ml of ice-cold ethanol were added to precipitate the DNA. The DNA was pelleted by centrifugation at 13,000×*g* for 30 min at 4°C. The pellet was washed two or three times with ice-cold 70% alcohol and dried in a vacuum. DNA was dissolved in 10 µl of ultrapure water and stored at –20°C until further use.

## Amplification of DNA

Different regions of the total genomic DNA isolated from the leaves of alder were subjected to amplification using PCR. Each amplification was carried out for 35 cycles using a thermal cycler (GeneAmp PCR2400, Perkin-Elmer). Each cycle comprised of 30 s of denaturation at 94°C, 30 s of annealing at the appropriate temperature and 30 s of elongation at 72°C. This was followed by an extension step for 3 min at 72°C at the end of the run.

The reaction mixture for amplification was prepared in strictly aseptic conditions under a UV hood. Care was taken to prevent contamination by exogenous DNA. A 25-µl mix was prepared for amplification in thin-walled PCR tubes. The reaction mix contained 2.5 µl of each primer (from 0.5 mM stocks), 2.5 µl of 10× PCR buffer (Bangalore Genei), 2.5 µl of the dNTP mix (1.25 mM), and 0.3 µl of *Taq* polymerase (5 U/µl, Bangalore Genei), and the rest of the volume was made up with ultrapure water. Each amplification run included a negative control. One microlitre of DNA was added to each of the tubes except for the negative control, to which 1 µl of ultrapure water was added instead. The primers FGPS 6 (5′-TGGAAAGCTTGATCCCTGGCT-3′; Normand et al. 1996)], FGPS 505′ [5′-GTATTACCGCGGCTGCTG-3′; Normand et al. 1996)], FGPS 485 [5′-CAGCAGCCGCGGTAA-3′; Normand et al. 1996)] and FGPS 910′ [5′-AGCCTTGCGGCCGTACTCCC-3′; Normand et al. 1996)] were used for amplification of the chloroplast 16S rRNA gene. The proximal part of the 16S rRNA gene was amplified using the primer pair FGPS 6 and FGPS 505′. Primer FGPS 6 is located at co-ordinate 6 and primer FGPS 505′ at position 505′ in the 16S rRNA gene of *Frankia* (Normand et al. 1996), but these primers could also be used to amplify the chloroplast 16S rRNA gene of *A. nepalensis* (see below). Samples of *A. nepalensis* genomic DNA were used as templates for PCR with these primers and the products were electrophoresed on 2% agarose gels. The nuclear 18S-28S region was amplified using the primers ITS1-PLANT (5′-CGCGAGAAGTCCACTG-3′; P. Normand, personal communication) and ITSC26A [5′-GTTTCTT TTCCTCCGCT-3′; Wen and Zimmer 1996)]. Primer AnpITS1 [5′-CCGCGAACCTGTCACAACAA-3′], designed in our laboratory, was used together with primer ITSC26A to amplify the 18S-28S ITS region. This primer is based on sequence analysis of part of the corresponding region in *A. nepalensis* (our unpublished data) and comparisons with sequences from alder and non-alder (*Corylus*, *Carpinus* and *Betula*) species retrieved from GenBank.

## AFLP analysis

The PCR products obtained from different samples under identical conditions of amplification could be distinguished by electrophoresis on agarose gels. The amplicons generated from different samples were electrophoresed at 5 V/cm for 4 h on agarose gels (1.5% for the 18S-28S ITS and 2% for the 16S rRNA gene) containing ethidium bromide. After electrophoresis the amplified products were visualised with GelDoc 1000 (BioRad) and the images were captured and saved on a floppy disc. The profiles were analysed using Multi Analyst software.

## Digestion of PCR products with restriction endonucleases

For restriction analysis 5–8 µl of amplicon was digested with 2 U of enzyme in a 20-µl mix prepared in a microcentrifuge tube, containing 2 µl of the appropriate buffer (10×); the remaining volume was made up with ultrapure water. The mix was incubated for 3 h at the optimal temperature recommended by the enzyme supplier. The restriction enzymes *Msp*I, *Mbo*I and *Scr*FI were

selected based on computer analysis of published alder sequences using MacVector software, and used for restriction analysis of different regions. The samples were then electrophoresed on 3% agarose gels at 40 V for 8 h. The gel was stained with ethidium bromide and the bands were visualised using the GelDoc 1000. The profiles were analysed using Multi-Analyst software. In cases where no digestion was observed, samples were digested with other restriction enzymes to rule out the possibility of inhibition by salt or polysaccharides.

### Assay for nitrogenase activity

Prior to the isolation of DNA, the nitrogenase activity was measured using the Acetylene Reduction Assay (ARA; Stewart et al. 1968) on the day the nodules were collected. The surface-sterilised nodules were dried on a filter paper. Fifty nodules from each tree were placed in a sterile vial and sealed with a stopper and Parafilm to make it airtight. Three replicates were taken for each tree. One millilitre of air in the vial was replaced with 1 ml of acetylene gas. A control containing acetylene gas without any nodules was also set up. The vials were incubated for 3 h at 28 ± 2°C. One millilitre of the gas mixture from the vial was injected into the injection port of a gas chromatograph (HP4980D) and the area of the ethylene peak was recorded. The injection port, oven and detector temperatures were 120, 90, and 175°C, respectively. The carrier gas was $N_2$ and the flow rates of $H_2$, air and $N_2$ were 50, 120 and 10 ml/min, respectively. Under these conditions of assay the retention time for ethylene was approximately 1.45 min and that of acetylene was 2.5 min. The nodules from each vial were weighed using a Sartorius balance and the respective fresh weights were recorded. The nitrogenase activity was expressed as nmoles of ethylene produced/g (fresh weight) nodules/h.

It was not possible to carry out the ARA studies for all one hundred trees on a single day. Therefore, in order to minimise the effects of changing environmental factors, all ARA experiments were conducted continuously over a period of two months during which the environmental conditions were fairly uniform. Collection and assay of each set of nodules were performed on the same day and the conditions from the time of collection of nodules to their nitrogenase activity assay were kept constant for all the samples, to ensure comparability of the data.

## Results and discussion

### Isolation of DNA

The use of young leaves for isolation and the addition of 3% PVPP resulted in the elimination of most of the phenolics and a clean preparation of DNA could be obtained. Electrophoresis of the DNA revealed a broad band of approximately 20 kb that was utilised for amplification using specific primers for different regions.

### Amplification profiles obtained with primers directed to the proximal part of the chloroplast 16S rRNA gene

The primers FGPS 6 and FGPS 505′, which were derived from the *Frankia* 16S rRNA sequence, were used to amplify part of the chloroplast 16S rRNA from *A. nepalensis*. The annealing temperature chosen for amplification was 45°C. At higher annealing temperatures no amplification products were obtained. To identify possible annealing sites for the primer pair, a homology search was done using MacVector software on the chloroplast 16S rDNA sequences from *A. incana* (Accession No. X68138) and *A. glutinosa* (Accession No. AF081529) retrieved from GenBank, since *A. nepalensis* sequences were not available. Primer FGPS 505′ was found precisely to match the sequence of the *A. incana* gene beginning at position 659 bp and the *A. glutinosa* gene at 420 bp, but less extensive matches were also detected elsewhere. Primer FGPS 6 did not exactly match either of these sequences. The best match was found to a site in the *A. incana* sequence beginning at position 180 (Table 1). Given the relatively low annealing temperature used, priming at alternative sites could explain the appearance of multiple bands in the profiles (see below). Very faint bands were not considered in classifying the profiles. However, we cannot exclude the possibility that some of the weaker bands are due to mispriming elsewhere in the total DNA. This region of the 16S rRNA gene has also been reported to be more variable than other segments of the sequence in microorganisms (Harry et al. 1991).

The volume of amplified product loaded on the gel was kept constant. The amounts of amplified product obtained from different samples varied, as revealed by the intensity of the bands on the gel. This could be explained if the degree of complementarity between the template and primer varied from sample to sample. The reaction was repeated to confirm the amplification profiles of the samples in which amplification was weak. The possible presence of additional bands not revealed in the initial runs was excluded by loading a larger volume of the PCR product, thus ensuring the authenticity of the classification of samples based on the profiles obtained.

Analysis of all 100 samples revealed three different profiles (Fig. 1), designated 1, 2, and 3. The approximate sizes of the bands present in the different profiles were determined using the Multi-Analyst software. Samples with Profile 1 showed a single band of approximately 470 bp (the size expected based on the best primer

**Table 1** Annealing sites for primer FGPS 6 found in the chloroplast 16S rRNA genes of *Alnus glutinosa* and *A. incana*

| Species | Position | Extent of match | Sequence |
|---|---|---|---|
| *A. glutinosa* | 21 | 11/20 | TGGAAA caccacTtCCcGtC |
| | 494 | 11/20 | TcccAgGgcTcAa CCCTGGa |
| | 609 | 11/20 | gcGAAAGCactc TgCtgGGC |
| | 696 | 11/20 | gtaAAcGaTgGATa CtaGGC |
| | 924 | 10/20 | gGacAcaggTGgTg CaTGGC |
| *A. incana* | 40 | 13/20 | TGGAtgGCTatA TttCTGGg |
| | 180 | 17/20 | TGGAgAGtTcGA TCC-TGGCT |
| | 1757 | 12/20 | TGGAAgtCTTctTt CgTttC |

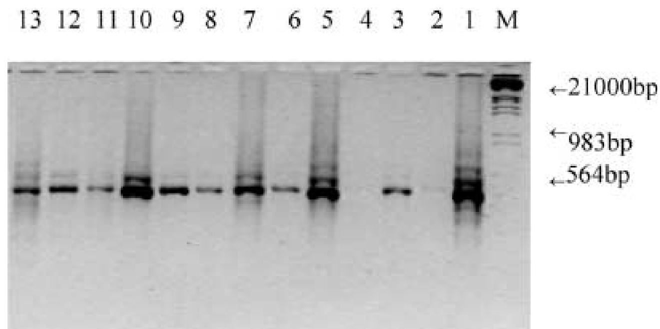


**Fig. 1** Profiles of the fragments amplified using primers located in the proximal part of the chloroplast 16S rRNA gene. Lane M, molecular-weight marker (λDNA/*Eco*RI + *Hind*III double digest). Lanes 2, 4 and 6, Profile 1; lanes 3, 8, 9, 11 and 12, Profile 2; lanes 1, 5, 7, 10 and 13, Profile 3

matches with the *A. incana* sequence). Profile 2 had a band of approximately 570 bp in addition to the 470-bp band, and Profile 3 had both of these, and a 660-bp band also.

## Amplification profiles obtained with primers for the central part of the chloroplast 16S rRNA gene

Primers FGPS 485 and FGPS 910' were utilised to amplify the central part of the chloroplast 16S rRNA gene at an annealing temperature of 45°C. Two bands of approximately 400 bp and 550 bp were visualised after electrophoresis on a 2% agarose gel. In an attempt to eliminate one of the bands, stringency conditions were increased by raising the annealing temperature to 50°C and then to 55°C, but even at 55°C both bands persisted. Comparative sequence analysis revealed that primer FGPS 485 completely matched the alder sequences but primer FGPS 910' could anneal only if a few mismatches were allowed. Under low-stringency conditions both primers could bind to alternative annealing sites. However, no differences were seen in the amplification profiles of different samples (Fig. 2), suggesting that this is a relatively less variable region.

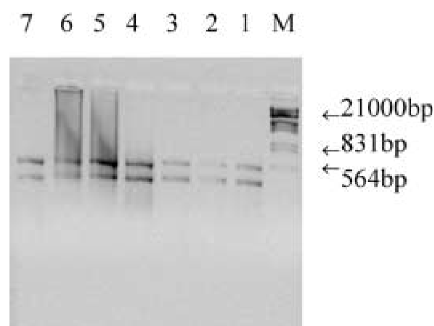


**Fig. 2** Profiles of the fragments amplified from the central part of the chloroplast 16S rRNA gene. Lane M, molecular-weight marker (λDNA/*Eco*RI + *Hind*III double digest). Lanes 1–7, amplicons obtained from different samples showing similar fragment profiles

## Amplification profiles obtained from the nuclear 18S-28S rDNA genes

Primer ITS1-PLANT, which anneals to the distal region of the 18S rRNA gene, and primer ITSC26A, complementary to the initial part of the 28S rRNA gene, were used to amplify the ITS I and ITS II flanking the 5.8S rRNA gene, at an annealing temperature of 45°C. Agarose gel electrophoresis revealed the expected single band of approximately 750 bp in some individuals, but profiles with multiple bands were also obtained. It was also noticed that the quantity of amplified product varied considerably. This made the interpretation of these profiles difficult. Nevertheless, it was possible to differentiate between different profiles based on some distinct bands. For example, the profiles shown in lanes 2, 3 and 5 of Fig. 3 were considered similar because of the presence of three major bands around 600 bp, 750 bp and 925 bp and one band at about 2000 bp. Raising the annealing temperature to 47°C failed to eliminate any of the extra bands. For ease of presentation, we include photographs of initial runs in which the volume of PCR products loaded in each lane was the same. Since the quantity of amplified product obtained differed for different samples, the amounts of DNA loaded may have been different. The profiles obtained for all the samples were compared after electrophoresis on 1.5% agarose gels. Nine different amplification profiles (A–I) could be distinguished (Figs. 3 and 4). Table 2 summarises the band composition of these profiles.

## RFLP analysis of the products amplified from the proximal part of the 16S rRNA gene

Restriction digestion of the amplicons obtained from the proximal part of the 16S rRNA gene was done using the four-base-cutter restriction endonuclease

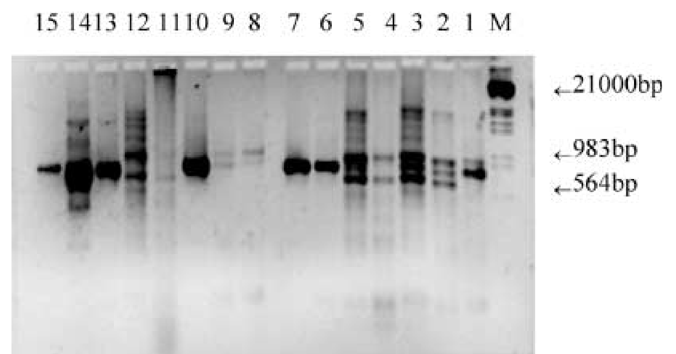


**Fig. 3** Fragment profiles obtained from the 18S-28S ITS region using the primers ITS1-PLANT/ITSC26A. Samples that yielded no products with these primers were treated as Profile I. Lane M, molecular-weight marker (see Fig. 1). Lanes 6, 7, 10, 13 and 15, Profile A. Lanes 2, 3 and 5, Profile B. Lane 12, Profile C. Lane 4, Profile D. Lanes 1, 8 and 9, Profile G. Lane 14, Profile H

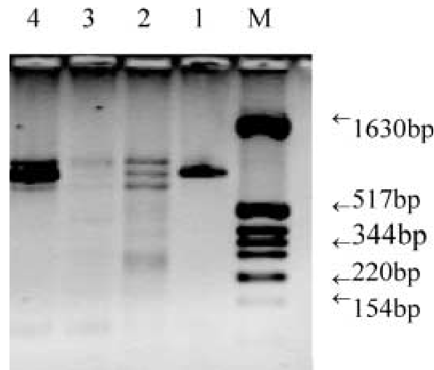


**Fig. 4** Fragment profiles obtained from the 18S-28S ITS region using the primers ITS1-PLANT/ITSC26A. Lane M, molecular-weight marker (pBR322 DNA *Hin*fI digest). Lane 1, Profile A. Lane 2, Profile E. Lanes 3 and 4, Profile F

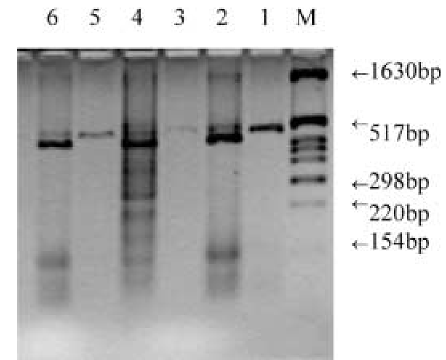


**Fig. 5** RFL profiles obtained from the proximal part of the 16S rRNA gene using the restriction endonuclease *Msp*I. Lane M, Molecular-weight marker (pBR322 DNA/*Hin*fI digest). Lanes 1, 3 and 5, undigested DNA from individual trees. Lanes 2 and 6, same samples as in lanes 1 and 5, digested with *Msp*I, and representing profile Ms(I)1. Lane 4, digested DNA representing profile Ms(I)2, obtained from the same sample as in lane 3

*Msp*I. After restriction digestion, electrophoresis was carried out on 3% agarose gels at 40 V for 8 h. Most of the samples gave rise to two bands of approximately 340 bp and 75 bp. Some samples yielded four additional bands of approximately 250 bp, 170 bp, 130 bp and 100 bp. A few samples were not cleaved. However, the same samples were susceptible to digestion by other restriction enzymes. Thus failure of *Msp*I to cleave these bands is likely to be due to the absence of the cognate recognition site. Hence, the profiles generated by restriction digestion of this region divide the alder trees into three groups. Those having the profiles Ms(I)1 (two bands) and Ms(I)2 (six bands) and a third group comprising samples which were not cleaved [Ms(I)3] (Fig. 5).

## RFLP analysis of the amplicons obtained from the central part of the 16S rRNA gene

For restriction analysis of the central part of the 16S rRNA gene two restriction endonucleases, *Mbo*I and *Msp*I, were used. When the two bands (of 400 and 550 bp) produced as a result of amplification of the middle part of the 16S rRNA gene of *A. nepalensis* were restricted with the enzyme *Mbo*I, bands of approximately 160 bp and 200 bp were obtained. The same samples, when digested with *Msp*I gave rise to three bands of approximately 135 bp, 230 bp and 340 bp. However, the restriction patterns obtained for different trees were similar (Fig. 6) and so could not be used for intraspecific differentiation of *A. nepalensis*.

## RFLP analysis of the products amplified from the 18S-28S ITS region

Non-coding regions of the genome normally show greater genetic variability since they are subject to less selection pressure than the coding regions. The internal transcribed spacer between the 18S and 28S rRNA sequences is one such region where the chances of detecting diversity are high. When this region was amplified using the primers ITS1-PLANT/ITSC26A, a great deal of variability was observed within the *A. nepalensis* population. However, when this region was amplified using primer AnpITS1 (instead of primer ITS1-PLANT; see Materials and methods) and primer ITSC26A, at an annealing temperature of 52°C, all the

**Table 2** Amplification profiles obtained for the 18S–28S *rrn* operon using the primers ITS1-PLANT and ITSC26A

| Profile | Approximate band size (bp)[a] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2000 | 1800 | 1550 | 1490 | 1210 | 1075 | 925 | 750 | 600 | 550 | 520 | 475 | 390 | 280 |
| A | – | – | – | – | – | – | – | + | – | – | – | – | – | – |
| B | + | + | + | – | + | – | + | + | + | + | + | – | – | – |
| C | + | + | + | – | + | – | + | – | + | + | + | – | – | – |
| D | – | – | – | – | – | – | + | – | + | – | – | + | + | + |
| E | – | – | – | – | – | – | + | + | + | – | – | + | + | + |
| F | – | – | – | – | – | – | + | + | + | – | – | – | – | – |
| G | – | – | – | – | – | – | + | + | – | – | – | – | – | – |
| H | – | – | – | + | – | + | – | + | – | – | – | + | – | – |
| I | – | – | – | – | – | – | – | – | – | – | – | – | – | – |

[a]Approximate band sizes were obtained using the Multi-Analyst software. +, band present; –, band absent

samples showed the same amplification profile. No differences in band size were detectable by agarose gel electrophoresis. To investigate this region in more detail and to search for differences between individuals, we carried out RFLP analysis of the amplicons. The enzyme used for restriction digestion of the amplified products was *Scr*F1. Restriction digestion of the amplicons was performed as described earlier. The xrestriction patterns of the different samples were resolved by agarose gel electrophoresis on 3% gels at 40 V for 8 h. The results obtained permitted the separation of the alder trees into nine different groups (Figs. 7, 8, 9 and 10). Table 3 shows the banding patterns of these profiles.

To predict the fragments that would be generated by digestion with *Scr*F1, the 18S-28S ITS sequences

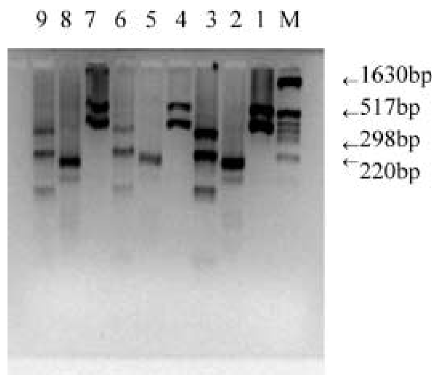


**Fig. 6** RFLP profiles for the central part of the 16S rRNA gene using the restriction endonucleases *Mbo*I and *Msp*I. Lane M, molecular-weight marker (pBR322 DNA/*Hin*fI digest). Lanes 1, 4 and 7, undigested DNA from individual trees. Lanes 2, 5 and 8, samples 1, 4 and 7 digested with *Mbo*I. Lanes 3, 6 and 9, samples 1, 4 and 7 digested with *Msp*I

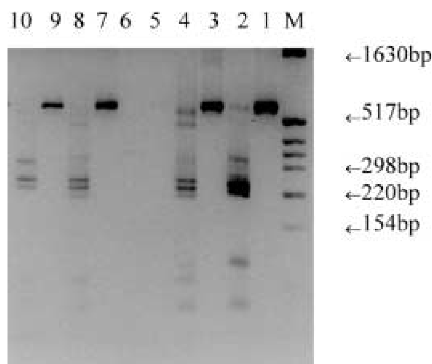


**Fig. 7** RFLP profiles obtained for the 18S-28S ITS region. Amplicons obtained with the primer pair AnpITS1/ITSC26A were digested with *Scr*F1. Amplicons that could not be digested were treated as Profile Sc9. Lane M, molecular weight marker (pBR322 DNA/*Hin*f1 digest). Lanes 1, 3, 7 and 9: undigested amplicons from individual trees. Lane 2, digested DNA sample 1, representing profile Sc4. Lanes 4 and 8, digested DNA samples 3 and 7, representing profile Sc3. Lane 10, digested DNA sample 9, representing profile Sc8

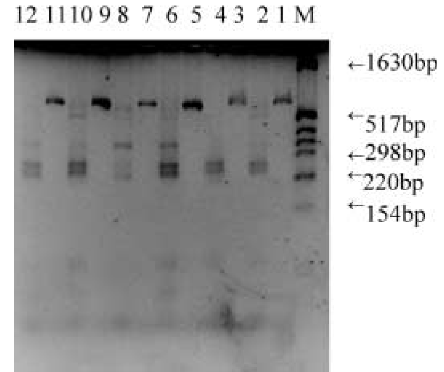


**Fig. 8** RFLP profiles obtained for the 18S-28S ITS region. For details see legend to Fig. 7. Lane M, molecular-weight marker (pBR-322 DNA/*Hin*fI digest). Lanes 1, 3, 5, 7, 9 and 11: undigested amplicons from individual trees. Lanes 2 and 4: digested samples 1 and 3, representing profile Sc6. Lanes 6, 8, 10 and 12: digested DNA samples 5, 7, 9 and 11, representing profile Sc2

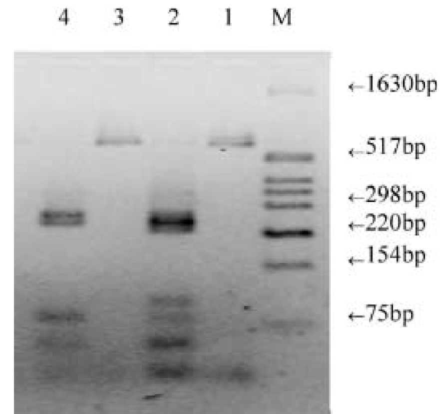


**Fig. 9** RFLP profiles obtained for the 18S-28S ITS region. For details see legend to Fig. 7. Lane M, molecular weight marker (pBR322 DNA/*Hin*fI digest). Lanes 1 and 3, undigested amplicons from individual trees. Lane 2, Digested DNA sample 1, representing profile Sc5. Lane 4, digested DNA sample 2, representing profile Sc6

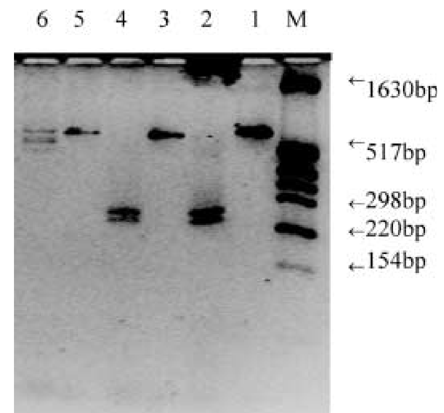


**Fig. 10** RFLP profiles obtained for the 18S-28S ITS region. For details see legend to Fig. 7. Lane M, molecular-weight marker (pBR322 DNA/*Hin*fI digest). Lanes 1, 3 and 5, undigested amplicons from individual trees. Lanes 2 and 4, digested DNA samples 1 and 3, representing profile Sc1. Lane 6, digested DNA sample 5, representing profile Sc7

**Table 3** RFLP profiles obtained for the 18S-28S rDNA ITS region using the restriction endonuclease *Scr*FI

| Profile | Approximate band size (bp)[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 630 | 520 | 330 | 265 | 245 | 180 | 130 | 110 | 60 | 20 |
| Sc1 | – | – | + | + | – | – | – | – | – | – |
| Sc2 | – | – | + | + | + | – | – | + | + | + |
| Sc3 | – | – | + | + | + | + | – | + | + | + |
| Sc4 | – | – | + | + | + | + | – | + | – | + |
| Sc5 | – | – | – | + | + | + | + | + | + | + |
| Sc6 | – | – | – | + | + | – | – | + | + | + |
| Sc7 | + | + | – | – | – | – | – | – | – | – |
| Sc8 | – | – | + | + | + | – | – | – | – | – |
| Sc9 | – | – | – | – | – | – | – | – | – | – |

[a]Approximate band sizes were determined using Multi-Analyst software. +, band present; -, band absent

available for *A. nepalensis* (our unpublished results) and *A. matsumurae* and were subjected to a computer analysis. The resulting restriction map predicted the generation of six bands of approximately 110 bp, 220 bp, 60 bp, 245 bp, 20 bp and 166 bp. Most of the bands represented in our profiles were compatible with this predictions. However, additional bands were also seen. This can be explained on the basis of the restriction map. If any of the predicted restriction sites were absent in the repeat unit, larger fragments than those predicted from the map would be obtained. However, if the 520 bp and 630 bp bands arise due to loss of restriction sites, the other smaller bands should not be present. This has indeed been observed in one of our profiles (Sc7). In some of the samples it was observed that the smaller bands and the bigger bands were present. This could be a consequence of either partial digestion or the presence of more than one type of rDNA repeat unit, which differ in sequence. A particular restriction site may be present in one of the repeat units but absent in another. However, this hypothesis remains to be proved. We have assumed here that the larger bands arise due to partial digestion, and they were therefore ignored in our analysis.

### Assay for nitrogenase activity

One hundred and fifty nodules were studied from each tree and their mean ARA values were calculated. A range of ARA values was obtained. A few trees at both sites showed very high nitrogenase activity. It was observed that relatively more of the trees at the NEHU Permanent Campus belonged to the high nitrogen-fixing group (Tables 4 and 5).

### Relationship between the PCR-AFL/RFL profiles and nitrogenase activity

The values obtained in the acetylene reduction assay, representing the nitrogenase activity, showed a wide range (Tables 4 and 5). To facilitate comparisons, the values were grouped into classes, with each class representing a range of ARA values (0–5, 5–10, 10–15, 15–20, 20–30, 30–40, 40–50, 50–60, 60–70, 70–80 and 80–90). Most trees fell in the range of 0–5 nmol ethylene produced/g nodule fresh wt./h, followed by those belonging to the range of 5–10 nmol ethylene produced/g nodule fresh wt./h (Fig. 11). Some 74% of trees had nitrogenase activity below 15 nmol ethylene produced/g nodule fresh wt/hr. A few trees showed very high nitrogenase activity. Trees having an ARA value exceeding 15 nmol ethylene produced/g nodule fresh wt/h were considered as high nitrogen fixers and those below this threshold were considered as low nitrogen fixers.

The AFL/RFL profiles were thoroughly scanned and efforts were made to correlate them with the ARA values. Tables 4 and 5 show the different PCR-AFLP/RFLP profiles for each tree. Most of the profiles were common to both high and low nitrogen-fixing groups. However, it was observed that AFL profile 3 for the initial part of the 16S rRNA gene predominantly occurred in trees with low nitrogen fixing activity. There was only one exception to this i.e., of the ten trees showing this profile only one tree (No. PC50) exhibited a high nitrogen fixation rate (38.63 nmol ethylene produced/g fresh wt./hr).

Comparison of the RFL profiles obtained for the 18S-28S ITS region to the nitrogenase activities of the trees revealed that Profile Sc3 occurred in twelve trees. All twelve had low ARA values, the lowest being 1.20 nmol ethylene produced/g fresh wt/h and the maximum was 12.35 nmol ethylene produced/g fresh wt/h. Profile Sc5 was unique to a single tree having a low ARA value. The other RFLP patterns obtained for this region could differentiate between trees but they were not strictly restricted to either of the nitrogen-fixing groups. Nevertheless, profile Sc3 can definitely be considered as a molecular marker to weed out the low nitrogen-fixing trees. Overall analysis, combining all the profiles for a particular tree, could not clearly differentiate the two nitrogen-fixing groups. However, one interesting observation was made. Trees PC 27 and PC 39 had very high nitrogenase activity, exceeding

**Table 4** Nitrogenase activities and AFLP/RFLP profiles of trees from Permanent Campus

| Sample No. | Nitrogenase activity[a] | AFLP profile with primers FGPS 6 and FGPS 505′ (proximal 16S) | *Msp*I RFLP profile[b] | AFLP profile with primers ITS1 and ITSC26A (18S-28S) | *Scr*FI RFLP profile[b] |
|---|---|---|---|---|---|
| PC1 | 2.53 ± 0.40 | 1 | Ms(I)1 | B | Sc6 |
| PC2 | 1.58 ± 0.29 | 1 | Ms(I)1 | I | Sc3 |
| PC3 | 55.02 ± 2.34 | 2 | Ms(I)1 | I | Sc2 |
| PC4 | 15.45 ± 1.35 | 2 | Ms(I)1 | A | Sc6 |
| PC5 | 17.79 ± 3.68 | 2 | Ms(I)3 | I | Sc6 |
| PC6 | 4.58 ± 0.63 | 2 | Ms(I)3 | A | Sc6 |
| PC7 | 12.35 ± 0.73 | 1 | Ms(I)1 | A | Sc3 |
| PC8 | 8.99 ± 3.09 | 3 | Ms(I)2 | I | Sc7 |
| PC9 | 35.63 ± 2.67 | 1 | Ms(I)1 | E | Sc9 |
| PC10 | 14.32 ± 1.71 | 1 | Ms(I)1 | B | Sc9 |
| PC11 | 12.48 ± 2.58 | 1 | Ms(I)1 | B | Sc6 |
| PC12 | 21.59 ± 1.11 | 2 | Ms(I)1 | B | Sc6 |
| PC13 | 5.98 ± 1.12 | 1 | Ms(I)3 | B | Sc6 |
| PC14 | 10.27 ± 1.45 | 1 | Ms(I)3 | B | Sc3 |
| PC15 | 3.96 ± 0.61 | 1 | Ms(I)1 | B | Sc2 |
| PC16 | 10.03 ± 1.95 | 2 | Ms(I)2 | B | Sc2 |
| PC17 | 1.61 ± 0.17 | 1 | Ms(I)1 | D | Sc6 |
| PC18 | 2.43 ± 0.51 | 1 | Ms(I)2 | C | Sc2 |
| PC19 | 1.72 ± 0.81 | 1 | Ms(I)1 | C | NA |
| PC20 | 10.87 ± 2.02 | 1 | Ms(I)1 | I | Sc2 |
| PC21 | 18.59 ± 1.36 | 2 | NA | B | Sc6 |
| PC22 | 20.16 ± 2.56 | 1 | Ms(I)1 | A | Sc9 |
| PC23 | 1.83 ± 0.13 | 1 | Ms(I)1 | H | Sc3 |
| PC24 | 5.46 ± 1.66 | 2 | Ms(I)1 | D | Sc6 |
| PC25 | 4.65 ± 1.96 | 2 | Ms(I)2 | D | Sc6 |
| PC26 | 12.31 ± 0.31 | 2 | Ms(I)1 | E | Sc9 |
| PC27 | 86.00 ± 3.24 | 1 | Ms(I)2 | I | Sc6 |
| PC28 | 62.09 ± 3.72 | 2 | Ms(I)2 | A | Sc6 |
| PC29 | 25.00 ± 2.26 | 1 | Ms(I)2 | I | Sc7 |
| PC30 | 6.74 ± 1.21 | 1 | Ms(I)1 | A | Sc6 |
| PC31 | 5.96 ± 1.40 | 2 | Ms(I)2 | I | Sc5 |
| PC32 | 7.52 ± 1.59 | 2 | Ms(I)2 | A | Sc6 |
| PC33 | 66.51 ± 2.41 | 2 | Ms(I)2 | A | Sc2 |
| PC34 | 6.60 ± 0.40 | 1 | Ms(I)2 | A | Sc2 |
| PC35 | 30.63 ± 1.50 | 1 | Ms(I)1 | I | Sc2 |
| PC36 | 8.75 ± 2.48 | 1 | Ms(I)1 | I | Sc6 |
| PC37 | 3.54 ± 1.08 | 1 | Ms(I)1 | I | Sc6 |
| PC38 | 82.28 ± 2.22 | 1 | Ms(I)2 | I | Sc2 |
| PC39 | 84.15 ± 2.21 | 1 | Ms(I)1 | B | Sc1 |
| PC40 | 2.41 ± 0.56 | 1 | Ms(I)1 | B | Sc6 |
| PC41 | 13.31 ± 0.47 | 2 | NA | I | Sc6 |
| PC42 | 6.25 ± 2.40 | 1 | Ms(I)2 | A | Sc1 |
| PC43 | 2.44 ± 0.20 | 1 | Ms(I)2 | A | Sc6 |
| PC44 | 5.28 ± 1.26 | 1 | Ms(I)2 | I | NA |
| PC45 | 2.11 ± 0.21 | 1 | Ms(I)2 | C | Sc1 |
| PC46 | 10.71 ± 1.91 | 1 | Ms(I)1 | D | Sc1 |
| PC47 | 21.50 ± 1.24 | 1 | NA | B | Sc2 |
| PC48 | 18.33 ± 1.52 | 1 | Ms(I)1 | E | Sc9 |
| PC49 | 10.31 ± 0.85 | 1 | Ms(I)1 | I | Sc2 |
| PC50 | 38.63 ± 2.57 | 3 | Ms(I)2 | I | Sc8 |

[a]Nitrogenase activity was determined by the acetylene reduction assay (see Materials and methods for details)
[b]NA, not available

80 nmol ethylene produced/g fresh wt/h. Each had a unique RFLP profile. The former had the profile [1, Ms(I)2, I, Sc6] and the latter [1, Ms(I)1, B, Sc1]. It is noteworthy that these two profile groups were very rare, each being present in only one tree out of the 100 screened. It is difficult to say at this stage whether the association of high nitrogenase activity with these profile groups is significant or not. Further analysis of a larger population of alder trees will be required to clinch the issue.

A question may be raised about the presence of different *Frankia* genotypes in the nodules (Diem et al. 1983; Clawson et al. 1998). We have taken this into account. One hundred and fifty nodules were randomly collected from each tree. Therefore, it is likely that the entire range of *Frankia* genotypes was repre-

**Table 5** Nitrogenase activities and AFLP/RFLP profiles of trees from Upper Shillong

| Sample No. | Nitrogenase activity[a] | AFLP profile with primers FGPS 6 and FGPS 505′ (proximal 16S) | *Msp*I RFLP profile[b] | AFLP profile with primers ITS1 and ITSC26A (18S-28S) | *Scr*FI RFLP profile[b] |
|---|---|---|---|---|---|
| US1 | 3.85 ± 0.72 | 3 | Ms(I)2 | G | Sc3 |
| US2 | 0.34 ± 0.07 | 3 | Ms(I)2 | B | Sc6 |
| US3 | 11.78 ± 1.79 | 1 | Ms(I)1 | B | Sc3 |
| US4 | 4.06 ± 1.40 | 2 | Ms(I)2 | D | NA |
| US5 | 2.56 ± 0.61 | 2 | Ms(I)1 | B | Sc3 |
| US6 | 3.89 ± 1.92 | 1 | Ms(I)1 | I | Sc2 |
| US7 | 0.65 ± 0.22 | 1 | Ms(I)2 | A | Sc7 |
| US8 | 6.03 ± 1.36 | 1 | Ms(I)2 | A | NA |
| US9 | 1.98 ± 0.51 | 1 | Ms(I)2 | G | Sc2 |
| US10 | 6.29 ± 1.89 | 3 | Ms(I)1 | G | Sc6 |
| US11 | 8.35 ± 2.28 | 3 | Ms(I)1 | I | Sc6 |
| US12 | 15.51 ± 0.21 | 1 | Ms(I)1 | A | Sc8 |
| US13 | 2.14 ± 0.91 | 1 | Ms(I)1 | D | Sc9 |
| US14 | 1.20 ± 0.27 | 1 | Ms(I)1 | C | Sc3 |
| US15 | 38.84 ± 1.06 | 1 | NA | A | Sc1 |
| US16 | 2.16 ± 0.72 | 2 | Ms(I)2 | I | Sc8 |
| US17 | 2.97 ± 0.09 | 1 | Ms(I)1 | H | NA |
| US18 | 2.16 ± 0.88 | 3 | Ms(I)2 | A | Sc2 |
| US19 | 2.04 ± 0.72 | 1 | Ms(I)2 | B | Sc1 |
| US20 | 1.31 ± 0.40 | 1 | Ms(I)2 | D | Sc4 |
| US21 | 6.36 ± 2.59 | 1 | Ms(I)1 | C | Sc8 |
| US22 | 10.13 ± 1.03 | 3 | Ms(I)2 | A | Sc1 |
| US23 | 6.95 ± 1.93 | 2 | Ms(I)1 | D | Sc3 |
| US24 | 0.46 ± 0.06 | 2 | Ms(I)2 | A | Sc2 |
| US25 | 9.22 ± 0.02 | 2 | Ms(I)1 | F | Sc1 |
| US26 | 1.23 ± 0.51 | 2 | Ms(I)1 | F | Sc1 |
| US27 | 7.76 ± 0.97 | NA | NA | D | Sc2 |
| US28 | 2.58 ± 0.86 | 2 | Ms(I)2 | I | Sc2 |
| US29 | 12.08 ± 1.25 | 2 | Ms(I)1 | G | Sc1 |
| US30 | 9.81 ± 1.15 | 2 | Ms(I)2 | G | Sc2 |
| US31 | 5.69 ± 1.40 | 3 | Ms(I)1 | D | Sc2 |
| US32 | 4.04 ± 1.19 | 2 | Ms(I)1 | D | Sc6 |
| US33 | 84.67 ± 2.19 | 1 | Ms(I)1 | D | Sc6 |
| US34 | 70.92 ± 4.47 | 2 | Ms(I)2 | F | Sc9 |
| US35 | 4.27 ± 2.04 | 1 | Ms(I)1 | A | Sc6 |
| US36 | 5.62 ± 0.44 | 3 | Ms(I)1 | A | Sc1 |
| US37 | 13.01 ± 2.50 | 1 | Ms(I)1 | NA | NA |
| US38 | 19.16 ± 1.48 | 1 | Ms(I)1 | NA | NA |
| US39 | 20.04 ± 1.50 | 2 | Ms(I)2 | B | Sc2 |
| US40 | 12.16 ± 0.85 | 2 | Ms(I)1 | B | Sc3 |
| US41 | 9.41 ± 0.58 | 2 | Ms(I)2 | E | Sc3 |
| US42 | 16.65 ± 0.36 | 2 | Ms(I)2 | I | Sc7 |
| US43 | 2.74 ± 0.65 | 2 | Ms(I)1 | I | Sc2 |
| US44 | 6.76 ± 0.94 | 2 | Ms(I)2 | F | NA |
| US45 | 5.90 ± 1.43 | 2 | NA | F | Sc3 |
| US46 | 3.12 ± 1.09 | 2 | Ms(I)1 | E | Sc2 |
| US47 | 8.45 ± 0.82 | 2 | Ms(I)2 | D | NA |
| US48 | 9.57 ± 1.54 | 2 | Ms(I)1 | F | NA |
| US49 | 39.70 ± 2.55 | 2 | Ms(I)2 | D | NA |
| US50 | 11.43 ± 2.05 | 2 | Ms(I)2 | D | Sc2 |

[a]Nitrogenase activity was determined by the acetylene reduction assay (see Materials and methods for details)
[b]NA, not available

sented. The average nitrogenase activity estimates, therefore, represented all the different *Frankia* genotypes associated with the given tree. Therefore, high or low nitrogenase activity is more likely to be a function of the genotype of the tree. Thus, the present study has been able to develop molecular markers that can be used to eliminate *A. nepalensis* genotypes that support low nitrogenase activity. In addition to this, the study also identified two profiles as likely markers for genotypes that support high nitrogenase activity.

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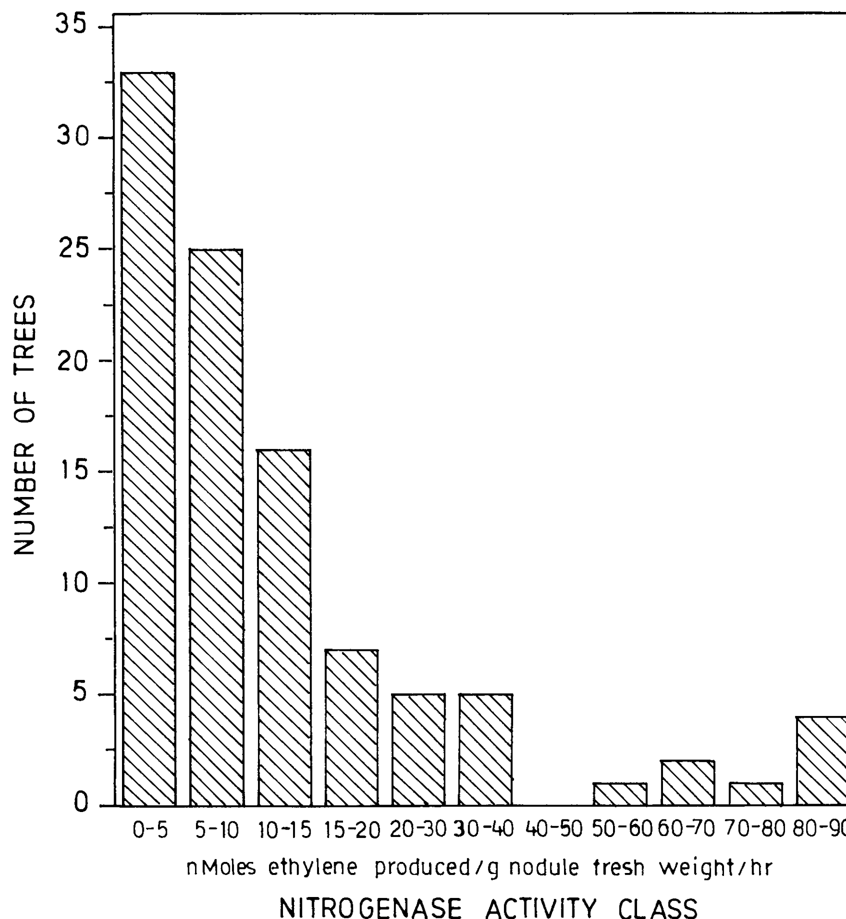


**Fig. 11** Graphical representation of the number of trees belonging to each nitrogenase activity class

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