Amplicon restriction patterns associated with nitrogenase activity of root nodules for selection of superior *Myrica* seedlings

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Trees of *Myrica* sp. grow abundantly in the forests of Meghalaya, India. These trees are actinorhizal and harbour nitrogen-fixing *Frankia* in their root nodules and contribute positively towards the enhancement of nitrogen status of forest areas. They can be used in rejuvenation of mine spoils and nitrogen-depleted fallow lands generated due to slash and burn agriculture practiced in the area. We have studied the association of amplicon restriction patterns (ARPs) of *Myrica* ribosomal RNA gene and internal transcribed spacer (ITS) region and nitrogenase activity of its root nodules. We found that ARPs thus obtained could be used as markers for early screening of seedlings that could support strains of *Frankia* that fix atmospheric nitrogen more efficiently.

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1. Introduction

Nitrogen-fixing actinomycete micro-symbiont *Frankia* nodulates roots of dicotyledonous plants belonging to 8 plant families and 23 genera known as actinorhizal genera. The eight plant families are Betulaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricaceae, Rhamnaceae and Rosaceae. Some of the genera commonly found in India include *Alnus, Casuarina, Coriaria, Elaeagnus, Hippophae and Myrica.*

Actinorhizal plants are multipurpose trees: they can be used for land reclamation and in regeneration of forests, and their wood can be used as timber and fuel. Actinorhizal trees such as *Alnus* have been used in the reclamation of mine spoils in Britain (Schwintzer and Tjepkema 1990), and *Alnus, Elaeagnus* and *Hippophae* have been widely used for land stabilization. Several actinorhizal trees like *Alnus* and *Casuarina* are used as timber and fuel wood. *Casuarina* trees are planted extensively as windbreaks and to stabilize dunes against wind erosion. Actinorhizal trees are also valuable for reclamation of mine spoils and rehabilitation of wastelands. They quietly contribute to the overall nitrogen status of forests. Members of some genera also find place in human diet and medicines. For example, *Hippophae rhamnoides* is cultivated for its berries for human consumption in Eastern Europe, India and China. Actinorhizal genus *Myrica* has also been exploited by traditional Indian medical system for its medicinal properties.

Myrica esculenta Buch.-Ham. ex D. Don belongs to the family Myricaceae and is commonly known as 'Box myrtle', '*Soh-Phie*' in local vernacular Khasi, '*Nagatenga*' in Assamese and '*Kaphal*' in Hindi. In addition to its medicinal value, the tree is useful in stabilizing nitrogen-depleted soils.

Two species of genus *Myrica* (*M. esculenta* and *M. nagi*) are found in Meghalaya, India (Yanthan *et al.* 2011). A significant role of the actinorhizal host plant in securing optimized symbiotic association with *Frankia* strains that fix atmospheric nitrogen more efficiently has been hypothesized (Verghese and Misra 2000; Chauhan and Misra 2002; Verghese and Misra 2002). Therefore, the present investigation was taken up for development of molecular markers based on amplicon restriction pattern (ARP) for early screening of seedlings of *Myrica* sp. that would support better symbiotic nitrogen fixation. Chauhan and Misra (2002) had found an association between ARP of *rrn* operon and

Keywords. Amplicon restriction pattern; molecular markers; *Myrica*; nitrogenase activity

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Table 1. Amplicon restriction patterns generated by restriction enzyme *Mbo*I

Profile	Samples	Size of restriction fragments (bp)						
		~800	~730	~700	~530	~380	~180	~90
PM1	NPC-1; NPC-3; NPC-4; NPC-6; NPC-7; NPC-9	−	−	+	+	−	+	+
PM2	NPC-2	+	+	−	+	+	+	+
PM3	NPC-5; NPC-8; NPC-10; NPC-11; NPC-13; NPC-14; NPC-17; NPC-20; NPC-21; NPC-22; NPC-23; NPC-24; NPC-25; NPC-27; NPC-29; NPC-30; NPC-31; NPC-32; NPC-33; NPC-34; NPC-35; NPC-36; NPC-37; NPC-38; NPC-39; NPC-40; NPC-41; NPC-42; NPC-43; NPC-44; NPC-45; NPC-46; NPC-47; NPC-48; NPC-49; NPC-50	−	−	−	+	−	+	+
PM4	NPC-12; NPC-15; NPC-18	−	+	−	+	+	+	+
PM5	NPC-16; NPC-19; NPC-26; NPC-28	−	+	−	+	−	+	+
PM6	NRF-1; NRF-2; NRF- 3; NRF-4; NRF-5; NRF-6; NRF-7; NRF-8; NRF-9; NRF-11; NRF-12; NRF-13; NRF-15; NRF-17; NRF-18; NRF-19; NRF-20; NRF-21; NRF-22; NRF-23; NRF-25; NRF-26; NRF-27; NRF-28; NRF-29; NRF-30; NRF-31; NRF-32; NRF-33; NRF-34; NRF-35; NRF-36; NRF-37; NRF-38; NRF-39; NRF-40; NRF-41; NRF-42; NRF-43; NRF-44; NRF-45; NRF-46; NRF-47; NRF-48; NRF-49; NRF-50; NRF-51; NRF-52; NRF-53; NRF-54; NRF-55; NRF-56; NRF-57; NRF-58; NRF-60; NRF-61	−	−	+	−	−	−	+
PM7	NRF-10	+	+	+	−	−	−	+
PM8	NRF-14; NRF-16; NRF-59	−	−	+	+	−	−	+
PM9	NRF-24	−	−	+	+	+	+	+

(+) and (−) indicate presence or absence of the given band.

Table 2. Amplicon restriction patterns generated by restriction enzyme *Sau*96I

Profile	Samples	Size of restriction fragments (bp)					
		~800	~600	~500	~310	~200	~100
PS0	NPC-5; NPC-6; NPC-8; NPC-9; NPC-10	+	−	−	−	−	−
PS1	NPC-1; NPC-2; NPC-3; NPC-4; NPC-7; NPC-11; NPC-12; NPC-13; NPC-14; NPC-15; NPC-16; NPC-17; NPC-18; NPC-19; NPC-20; NPC-21; NPC-22; NPC-23; NPC-24; NPC-25; NPC-26; NPC-27; NPC-28; NPC-29; NPC-30; NPC-31; NPC-32; NPC-33; NPC-34; NPC-35; NPC-36; NPC-37; NPC-38; NPC-39; NPC-40; NPC-41; NPC-42; NPC-43; NPC-44; NPC-45; NPC-46; NPC-47; NPC-48; NPC-49; NPC-50	+	+	−	−	+	−
PS2	NRF-1; NRF-2; NRF-3; NRF-4; NRF-5; NRF-6; NRF-7; NRF-8; NRF-9; NRF-10; NRF-11; NRF-22; NRF-23; NRF-25; NRF-26; NRF-27; NRF-27; NRF-29; NRF-30; NRF-33; NRF-35; NRF-36; NRF-37; NRF-38; NRF-39; NRF-40; NRF-41; NRF-44; NRF-45; NRF-46; NRF-47; NRF-48; NRF-52; NRF-53; NRF-54; NRF-57; NRF-58; NRF-60	−	−	+	−	+	+
PS3	NRF-12; NRF-13; NRF-14; NRF-15; NRF-16; NRF-17; NRF-18; NRF-19; NRF-20; NRF-34; NRF-59	+	+	+	+	+	+
PS4	NRF-21; NRF-24; NRF-42; NRF-43; NRF-49; NRF-50; NRF-51; NRF-55;	−	−	+	+	+	+
PS5	NRF-31; NRF-32; NRF-61	+	−	+	−	+	+
PS6	NRF-56	−	+	+	+	+	+

Presence (+) or absence (−) of bands for each profile is indicated.

nitrogenase activity of root nodules in *Alnus nepalensis*. Therefore, we investigated to know whether a similar association also exists in *Myrica*.

2. Materials and methods

Two different sites were selected for collection of samples. First site was located within the campus of North-Eastern Hill University, at 25.36° N latitude, 91.53° E longitude at 1431 m above mean sea level. The second site was located about 35 km away in Nongkrem forest, at 25.50° N latitude, 91.88° E longitude at 1631 m above mean sea level. Soil samples were collected within a periphery of about 1 m from the base of each of the 10 trees randomly demarcated at each site. Debris present above the top soil was first cleared off and soil was then collected at a depth of 30 cm from the ground level using a 10×3 cm cylindrical soil corer. The collected soil was brought to the laboratory and air-dried at room temperature for 2 to 3 days until it was completely dried. The soil was then sieved through a 0.2 mm porous net and the weight recorded. Soil nitrogen was estimated for each soil sample separately using Kjeldahl method (Jackson 1967).

Fifty naturally growing fruit-bearing *Myrica nagi* trees were randomly selected at site 1, and 61 *Myrica esculenta* trees were randomly selected at site 2. There were two morphological variants at site 2. Fifty selected trees were one type, and 11 were the other type. Accordingly, we labelled *Myrica nagi* trees as morphotype-1, and the two variants of *Myrica esculenta* at site two as morphotype-2 and morphotype-3, respectively. *M. esculenta* trees were not found naturally growing at site 1, nor were any *M. nagi* trees found at site 2. From each tree, young, uninfected fresh leaves were collected into labelled plastic bags, brought to the laboratory and stored at −80°C for further studies. Isolation of DNA from the leaves was performed using the modified CTAB method (Dellaporta *et al.* 1983) as described earlier (Chauhan and Misra 2002).

Amplification for the 18S-28S rDNA ITS region and 18S rDNA of the isolated DNA was carried out for all the collected leaf samples using the Polymerase Chain Reaction (PCR). PCR was performed using either Applied Biosystems GeneAmp 9700Gold or Perkin Elmer GeneAmp 2400. Each reaction mix contained 2.5 µL of 10× PCR assay buffer (Bangalore Genei, India), 2.5 µL each of the primer (5 pmol), 2.5 µL of $MgCl_2$ (25 mM), 0.75 µL of *Taq* polymerase (3 Units/µL) and 2.5 µL each of the dNTPs (1.25 mM) and 1 µL of target DNA (approximately 10 ng DNA). Final volume was made to 25 µL by adding ultra pure water. Negative control was kept without addition of any DNA in the reaction mix. A total of 35 cycles were run for each reaction. An initial denaturation step of 10 min at 94°C was followed by 35 cycles of denaturation at 94°C for 1 min, 1 min of primer annealing at the appropriate temperature and 1 min of nucleotide extension at 72°C. Final-elongation step of 7 min at 72°C was allowed at the end of the PCR.

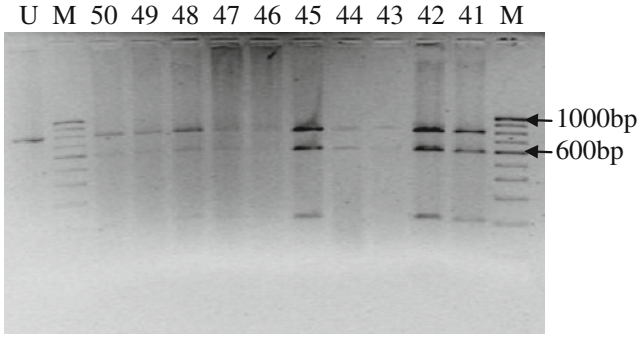

Figure 1. Amplicon restriction pattern (ARP) of morphotype-1 trees (samples 41–50) using S*au*96I. M, 100 bp DNA marker; U, undigested control DNA.

Forward primer ITSI PLANT (Navarro *et al.* 2003) located at the distal part of the 18S rRNA gene and the reverse primer ITSC26A (Wen and Zimmer 1996) located at the initial part of the 28S rRNA gene were used for amplification of the 18S-ITS1-5.8S-ITS2-28S rDNA internal transcribed spacer (ITS) region. Three amplicons were sequenced for validation. Sequence data were used for BLAST search in GenBank data base. Query sequences were aligned with retrieved sequences and analysed using CLUSTAL W (version 1.83).

The amplified 18S-28S rDNA ITS region was subjected to restriction digestion using restriction enzymes *Mbo*I and *Sau*96I selected on the basis of mock computer digestion using Mac Vector® software. The digestion mixture was prepared in a 1.5 mL tube containing 10 µL (app 50 ng) of the amplicon, 0.5 µL of the enzyme, 2 µL of the buffer and the final volume was adjusted to 20 µL by addition of requisite amount of pure water.

Digested amplicons were electrophoresed in 2% agarose gel. Twenty µL of each restriction product was mixed with 10 µL of loading buffer and loaded into separate wells of the gel. Electrophoresis was carried out for about 6–7 h at 45 V. Observation and quantification of the restriction fragments

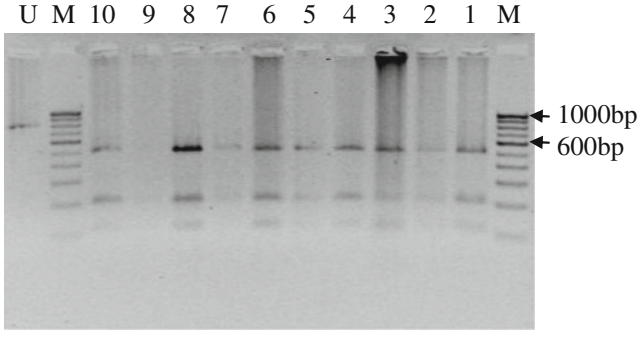

Figure 2. Amplicon restriction pattern (ARP) of morphotype-2 trees (samples 1–10) using *Sau*96I. M, 100 bp DNA marker; U, undigested control DNA.

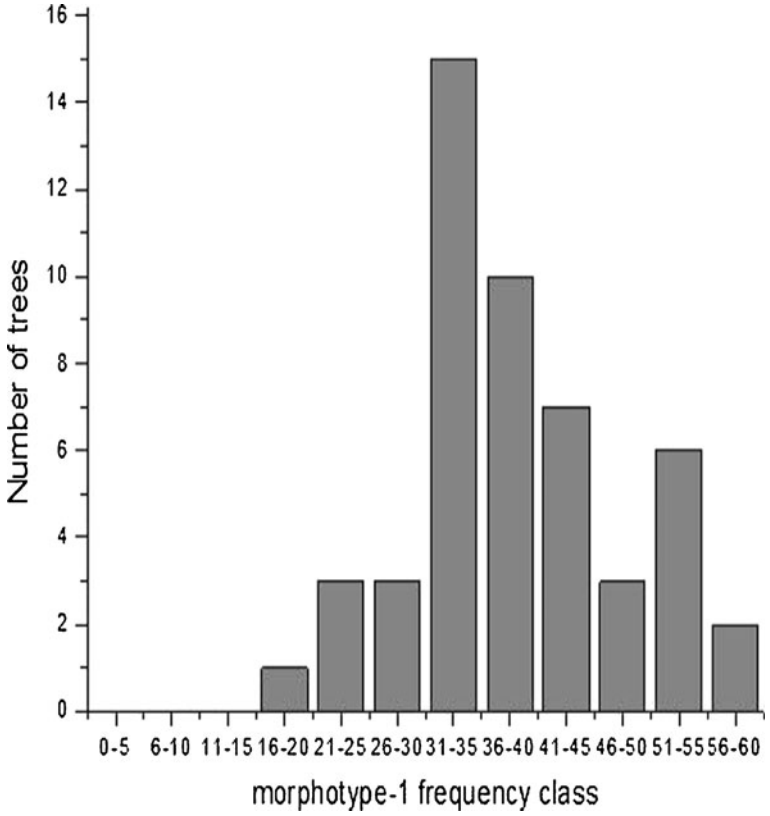

Figure 3. Graphic representation of frequency distribution of morphotype-1 (*Myrica nagi*) trees at NEHU campus based on their ARA activity (nmol C_2H_4 produced /mg nodule fresh wt/h).

was done using BioRad® Gel Doc 1000 with MultiAnalyst software or KODAK® Gel Logic 1500 Imaging System.

Nodules were collected from all the hundred and eleven trees at both the collection sites. Before collecting nodules the upper thin layer of soil just below each tree was first cleared off and the roots containing the nodules were traced to their respective trees in order to eliminate the chances of collecting nodules from a different tree. Nitrogenase activity of the freshly collected nodules was estimated on the day of their collection using the acetylene reduction assay (ARA) (Stewart *et al.* 1968). In order to draw a valid comparative inference, the time of collection of nodules till the estimation of ARA was maintained uniformly for all the samples. The ARA values for all the hundred and eleven trees were estimated with three replicates each.

The quantity of ethylene produced was measured using a Gas Chromatograph (model HP 4890D) equipped with flame ionization detector (FID). The injection port, oven and detector temperatures were maintained at 120, 90 and 175°C, respectively. Nitrogen was used as carrier gas and the flow rates of hydrogen, air and nitrogen were set at 50, 120 and 10 mL/min, respectively. The nitrogenase activity was estimated in terms of nmoles of ethylene produced per milligram fresh weight of nodule per hour.

3. Results and discussion

Constant voltage electrophoresis in 0.8% agarose gel showed high-molecular-weight genomic DNA bands for all the samples under study. Best amplification was obtained at annealing temperature of 49°C, where a single band of approximately 800 bp was obtained. Three amplicons, ME1, ME2 and ME3 were sequenced as described earlier (Yanthan *et al.* 2011) and deposited with the GenBank (accession numbers FJ469992, FJ469993 and FJ469994, respectively). BLAST search of GenBank using these sequences as query sequences retrieved 18S-ITS-28S rDNA sequences. The sequence homologies of the query sequences with the rDNA ITS sequences retrieved ranged between 94% and 100%. This confirmed that our amplicons belonged to the target nuclear rDNA segment.

Nine different profiles, PM1, PM2, PM3, PM4, PM5, PM6, PM7, PM8 and PM9, were obtained using restriction enzyme *Mbo*I (table 1). Five of these profiles (PM1, PM2,

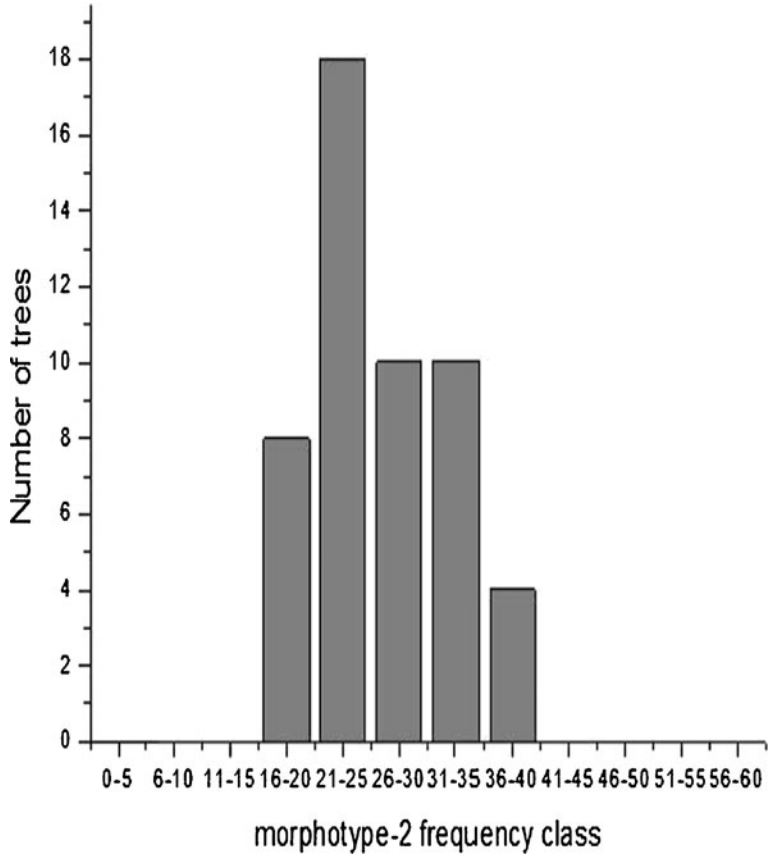

Figure 4. Graphic representation of frequency distribution of morphotype-2 (*Myrica esculenta*) trees at Nongkrem forest based on their ARA activity (nmol C_2H_4 produced/mg nodule fresh wt/h).

PM3, PM4 and PM5) were found only for *M. nagi* (morphotype-1) trees. The profiles PM6, PM7, PM8 and PM9 were found only for *M. esculenta* (morphotype-2 and morphotype-3) trees.

*Sau*96I digestion of all the samples revealed seven different profiles (table 2). Profiles PS1 and PS0 were confined to *M. nagi* trees (figure 1). The other profiles, PS2, PS3, PS4, PS5 and PS6, were found only for *M. esculenta* trees (figure 2).

It was observed that most of the *M. nagi* trees fell in the range of 31–35 nmol C_2H_4 produced/mg fresh wt/h frequency class (figure 3). Whereas the majority of *M. esculenta* trees fell in the range of 21–25 nmol C_2H_4 produced/mg fresh wt/h frequency class (figures 4 and 5). It was also found that the soil nitrogen at NEHU campus (site 1) ranged from 0.045% to 0.496% with a mean value of 0.178% and that at the Nongkrem forest (site 2) it ranged from 0.408% to 1.036% with a mean value of 0.628%. The difference between the two means was statistically significant. Average ARA was found to be higher for *M. nagi* trees collected from NEHU campus compared to *M. esculenta* trees collected from Nongkrem forest. It is likely that poorer nitrogen status of soil at NEHU campus could be responsible for higher nitrogenase activity exhibited by *Myrica nagi* trees.

The ARP of each tree was related with its respective Acetylene Reduction Assay (ARA) value in order to look for profiles which would serve as molecular markers for screening out trees belonging to high or low nitrogenase activity (supplementary table 1). We confined our comparisons among trees belonging to one species at a time. We could not compare trees belonging to the two different species firstly because they were collected from two different sites with significantly different levels of soil nitrogen, and secondly because the ARPs were species specific. A frequency class distribution of the samples in regard to their nitrogenase activity was prepared to make the comparisons easier. We considered trees with nodules showing higher than 35 nmol ethylene produced/mg fresh wt/hr as superior nitrogen fixers.

Since restriction profiles generated by either of the enzymes in isolation did not show any conclusive association with nitrogenase activity, we resorted to the multi-site

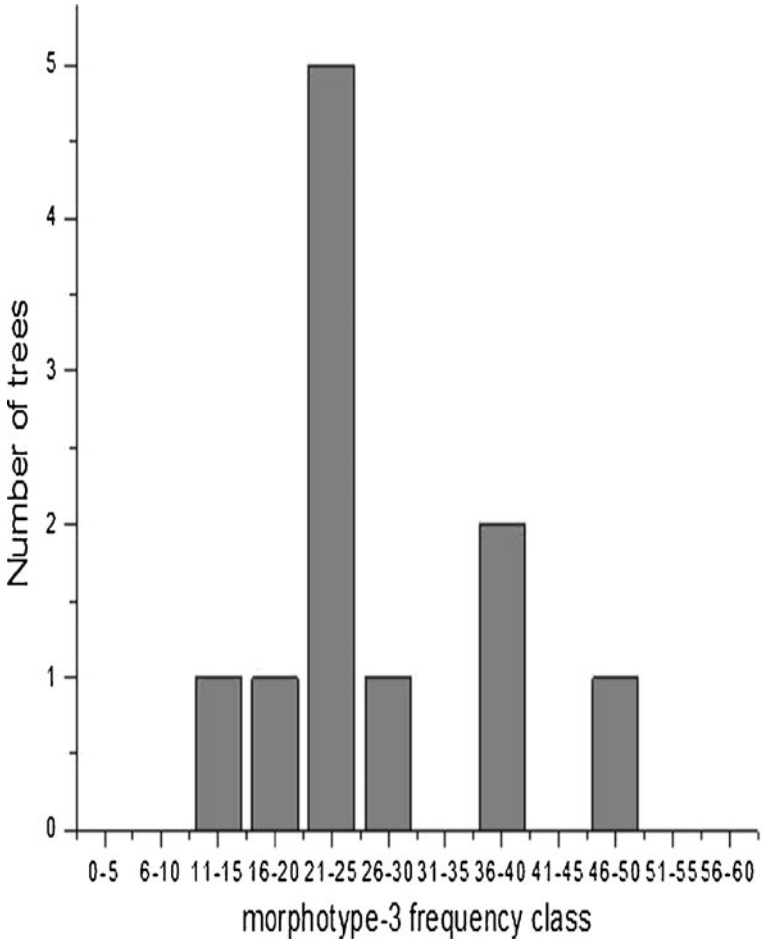

Figure 5. Graphic representation of frequency distribution of morphotype-3 (*Myrica esculenta*) trees at Nongkrem forest based on their ARA activity (nmol C_2H_4 produced/mg nodule fresh wt/h).

marker approach described earlier (Verghese and Misra 2000; Chauhan and Misra 2002). *M. nagi* samples NPC-5, NPC-8 and NPC-10 with relatively high nitrogenase activity shared the multi-site marker PM3:PS0 (supplementary table 1). This marker was not found in any tree with low nitrogenase activity. Therefore, it may be considered as a molecular marker to select *M. nagi* trees with ability to support higher nitrogenase activity in their root nodules.

M. esculenta samples NRF:21, NRF:42, NRF:43, NRF:49, NRF:50, NRF:51 and NRF:55 recorded nitrogenase activity ranging from 18.95 to 34.16 nmol of ethylene produced/mg fresh wt/h (supplementary table 1). These samples shared the profile combination PM6:PS4. This profile combination was not found in any tree with higher nitrogenase activity. Therefore, the profile combination PM6:PS4 could be considered as a marker to weed out *M. esculenta* trees with average to low nitrogenase activity. Although this marker could not weed out all low nitrogenase activity supporting trees, it may serve as one tool for selection of seedlings at nursery stage.

We do understand that nitrogenase activity in a symbiotic association is a complex trait. Nevertheless, we have found some association with the ARPs even for an unrelated DNA segment. A similar association was found by Chauhan and Misra (2002) in *Alnus nepalensis*. Therefore, we feel that the host genes involved in selection of more efficient *Frankia* strains for nodulation are linked to the *rrn* operon. It is likely that such gene(s) are present on the chromosome with nucleolar organizer. We argue that the ARP pattern would be useful for screening of *Myrica* seedlings at the nursery level for soil regeneration programmes.

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