

Hypervariable spacer regions are good sites for developing specific PCR-RFLP markers and PCR primers for screening actinorhizal symbionts

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While the ribosomal RNA like highly conserved genes are good molecular chronometers for establishing phylogenetic relationships, they can also be useful in securing the amplification of adjoining hyper-variable regions. These regions can then be used for developing specific PCR primers or PCR-RFL profiles to be used as molecular markers. We report here the use of ITS region of *rrn* operon of *Frankia* for developing PCR-RFL profiles capable of discriminating between closely related frankiae. We have also made use of the ITS1 region of the nuclear *rrn* operon of *Alnus nepalensis* (D Don) for designing a PCR primer for specific amplification of nuclear DNA of this tree.

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1. Introduction

Arrival of the polymerase chain reaction as a technique has opened the exciting era of designing specific primers that have applications in various fields of science like molecular diagnostics, forensic science and marker-assisted breeding. We have been making use of this technique in conjunction with restriction digestion of DNA with the intent of developing marker-assisted system for improvement of actinorhizal symbiosis (Varghese and Misra 2000; Chauhan and Misra 2002). Actinorhizal trees enter into a nitrogen-fixing symbiosis with the bacterium *Frankia* (Actinomycete: Frankiaceae). More than 200 dicotyledonous plants belonging to 8 families and 25 genera are known to harbour *Frankia* as a microsymbiont that fixes atmospheric nitrogen. These plants are important components of forest eco-systems.

India is endowed with varied climates extending from the deserts of west to heavy rainfall areas of northeast. The mountains of north are home to many actinorhizal plants. The plains and coastal regions of the south have *Casuarina* growing in abundance. Some actinorhizal

genera are present in the hills of south India as well. The actinorhizal genera represented in India include *Alnus*, *Casuarina*, *Coriaria*, *Elaeagnus*, *Hippophae* and *Myrica*. *Alnus* (Family Betulaceae) is a widely spread genus of the actinorhizal trees. Although about 47 species of *Alnus* are known (Swensen and Mullin 1997), only two species (*A. nepalensis* and *A. nitida*) are found growing naturally in India. They are distributed in the eastern and the western Himalaya respectively. Only one species of *Casuarina* (*C. equisetifolia*) is found in the plains and coastal regions of the country.

The initial work with root nodules of *Alnus nepalensis* D Don enabled development of *Frankia* genus-specific PCR primers (Simonet *et al* 1991). This was followed by development of more specific primers designed by Bosco *et al* (1992). Thus, it became very easy to amplify the *rrn* operon of *Frankia* from crushed root nodules of its host. Consequently, considerable work was done to establish phylogenetic relationship among *Frankia* infecting different hosts (Nazaret *et al* 1991; Nick *et al* 1992; Mirza *et al* 1994; Benson *et al* 1996; Normand *et al* 1996; Wolters *et al* 1997; Jeong *et al* 1999; Varghese *et al*

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2003). In all these investigations, the highly conserved 16S rRNA gene became handy. Subsequently, the use of primer pairs located in the 16S rRNA gene and the adjacent 23S rRNA gene enabled amplification of the *rrn* operon's internal transcribed spacer (ITS) regions (Jamann *et al* 1993; Rouvier *et al* 1996). While the 16S and 23S rRNA genes are highly conserved because of their importance in the survival of the organisms, the ITS regions are able to retain the sequence changes more effectively. Therefore, for studying the relationship among organisms at the inter-species level, the more conserved genes are useful, but for differentiating between different organisms at the sub-species level, it is the variable ITS region which is more useful.

Our work with *Frankia*, found associated with *Alnus nepalensis*, showed substantial variability for this organism (Ganesh *et al* 1994). We used the 16S rRNA gene for discriminating between different isolates of *Frankia* (Varghese and Misra 2000; Sarma and Misra 2002). In the present investigation we are reporting the use of *rrn* operon's ITS region for developing molecular markers with greater resolving power.

Any symbiotic association has at least two players. Bulk of the studies on the actinorhizal symbiosis have been confined to studying only one of the organisms involved in the association – either the microsymbiont *Frankia* or the host. Our approach is to study both the microsymbiont and the host. To start with, we have decided to concentrate on the alder-*Frankia* symbiosis. It is towards this aim that we decided to design a PCR primer that could selectively amplify only alder DNA. Once again, we thought it was more likely to succeed if the more variable ITS region could be utilized for the purpose. Once we have the necessary tools for both the host and the microsymbiont, we can use them for improving the symbiotic association in a holistic manner. Tagging of both the host and the microsymbiont will help in selecting the 'made for each other' combinations.

2. Materials and methods

2.1 Collection of samples

Root nodules were collected from different locations in India and one location in Germany (table 1). Three species of *Alnus* were represented – *A. nepalensis*, *A. nitida* and *A. glutinosa*. For comparison, a *Frankia* strain ACN1^{AG} (kindly given by Dr Philippe Normand, Université Claude-Bernard, Lyon 1, France), was also used. All the nodule samples were brought to the lab on ice.

For studies with the host, *A. nepalensis*, young leaves were collected from trees growing around Shillong. At each site fifty *A. nepalensis* trees were randomly selected for study. The leaves showed luxurious growth for major

part of the year except for the winter season (December–February), when there was maximum leaf fall. Young light green leaves were selected for molecular analysis. Bigger leaves at times showed fungal infection and were avoided. The same approach was used for collecting leaves of *Betula*, *Carpinus* and *Myrica*.

2.2 DNA isolation

DNA was extracted from individual nodule lobes, *Frankia* culture and leaves of alder, *Betula*, *Carpinus* and *Myrica* separately as described earlier (Varghese *et al* 2003). Fungal DNA was included as control to discount the possibility of amplification of pathogenic/associative fungal DNA. Fungal DNA was isolated from a mixed culture of fungi by rapid sonication of fungal culture in an eppendorf tube. This was followed by chloroform : isoamyl alcohol (24 : 1) extraction and ethanol precipitation (Chauhan 2000).

2.3 DNA amplification and restriction digestion

For *Frankia rrn* operon, DNA was amplified as described earlier (Varghese *et al* 2003). Amplified 16S-23S rDNA ITS products were digested using appropriate restriction enzymes. Four µl (approximately 100 ng) of the gel-purified PCR products were digested for 2 h in 10 µl final volume with 4 units of each enzyme respectively. PCR products for 16S-23S rDNA ITS were digested with restriction enzymes *TaqI*, *RsaI*, *AvaI* and *HinfI*. Buffers and temperatures were used as specified by the manufacturers for each endonuclease. Restriction-digested fragments were electrophoresed in 4% (w/v) agarose gel containing ethidium bromide. To detect the smaller fragments generated by restriction analysis, silver staining method was also employed as described below.

Restriction-digested products were first run in 8% acrylamide gel under denaturing condition. Gel was then stuck to glass plate and fixed in 10% acetic acid for 20 min after which, it was washed twice using distilled water for 5 min. It was then immersed into the silver staining solution [25 ml of 1% (w/v) silver nitrate solution; 0.25 ml of 37% (w/v) formaldehyde; water to make up the volume to 250 ml] for 20 min. After washing in distilled water for about 10 s, the gel was immersed in developing solution [6.25 g sodium carbonate; 0.25 ml of 37% (w/v) formaldehyde; water to make the volume 250 ml] for 5 min. The reaction was stopped by immersing in stop solution (10 mM EDTA-Na₂·2H₂O in distilled water) for 5 min. For preserving the gel on the glass plates, it was air-dried overnight after immersing for 1 h in a solution containing 75 ml of ethanol and 11.5 ml of glycerol. It proved to be a sensitive method detecting even the

Table 1. Nodule samples and sites of their collection for studies on *Frankia* amplicons.

Country	Province	Site	Host	Code assigned
India	Arunachal Pradesh	Hapoli	<i>Alnus nepalensis</i>	AnpHR
	Meghalaya	Upper Shillong	<i>Alnus nepalensis</i>	AnpUSR
		Nonkrem hills	<i>Alnus nepalensis</i>	AnpNHR
		Ooty	<i>Alnus nepalensis</i>	AnpOR
	Himachal Pradesh	Kulu	<i>Alnus nitida</i>	AntKR
Germany		Tuebingen	<i>Alnus glutinosa</i>	AgTR

smallest fragments of less than 50 bp in size. The bands generated were of high resolution and sharp.

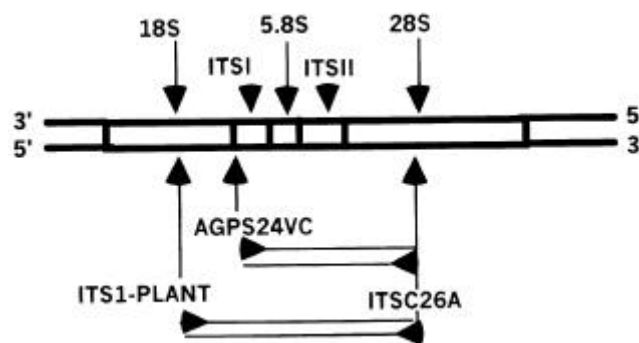
For securing the amplification of alder DNA, universal primers ITS1-PLANT (5'CGCGAGAAGTCCACTG3'; P Normand, personal communication) and ITSC26A (5'GTTTCTTTTCTCCGCT3', Wen and Zimmer 1996) were used. Since, ITS1-PLANT is located in the nuclear 18S rRNA gene and ITSC26A is located in the nuclear 28S rRNA gene, the two primers yielded the amplicon including the distal part of 18S rRNA gene, the ITS I, the 5-8S rRNA gene, the ITS II and the initial part of the 28S rRNA gene (figure 1).

2.4 Nucleotide sequencing and analysis

The nuclear 18S-28S ITS, on amplification using primer pair ITS1-PLANT/ITSC26A, showed multiple bands including the expected 750 base pair band. The band of interest was excised out and purified using the protocol of Byrnes *et al* (1995). These samples were then packed in gel ice and sent to M/S Bangalore Genei, India, for sequencing. The sequencing was done using an automated DNA sequencer that uses fluorescent label dye terminators or fluorescent label primers. The ABI's Ampli Taq FS dye terminator cycle sequencing chemistry, based on Sanger's dideoxy chain termination method (Sanger *et al* 1977) was used. These sequences were used for retrieving similar sequences from the GenBank data bank and compared for sequence similarity (Varghese *et al* 2003).

2.5 Designing alder specific primer

Aligned sequences of the 18S-28S ITS region (including distal part of the 18S rRNA gene, the 5-8S rRNA and the initial part of 28S rRNA, figure 1) of alder (*Alnus nepalensis*, *Alnus incana*, *Alnus crispa*, *Alnus glutinosa*, *Alnus matsumurae*), *Betula*, *Carpinus* and *Corylus* were compared. A region showing similarities between different species of alder and differences among the different genera, was selected to design a genus-specific probe for *Alnus*. To confirm if the primer designed could give alder-specific amplification, we decided to test amplification of

**Figure 1.** Organisation of the *rrn* operon of alder showing the locations of the PCR primers.

the DNA of other actinorhizal trees and some members of the alder family, Betulaceae, using these primers. This was done since *Alnus* is an actinorhizal genus belonging to family Betulaceae. Two actinorhizal trees *Elaeagnus* and *Myrica* and two members of the family Betulaceae, *Betula* and *Carpinus*, were tested.

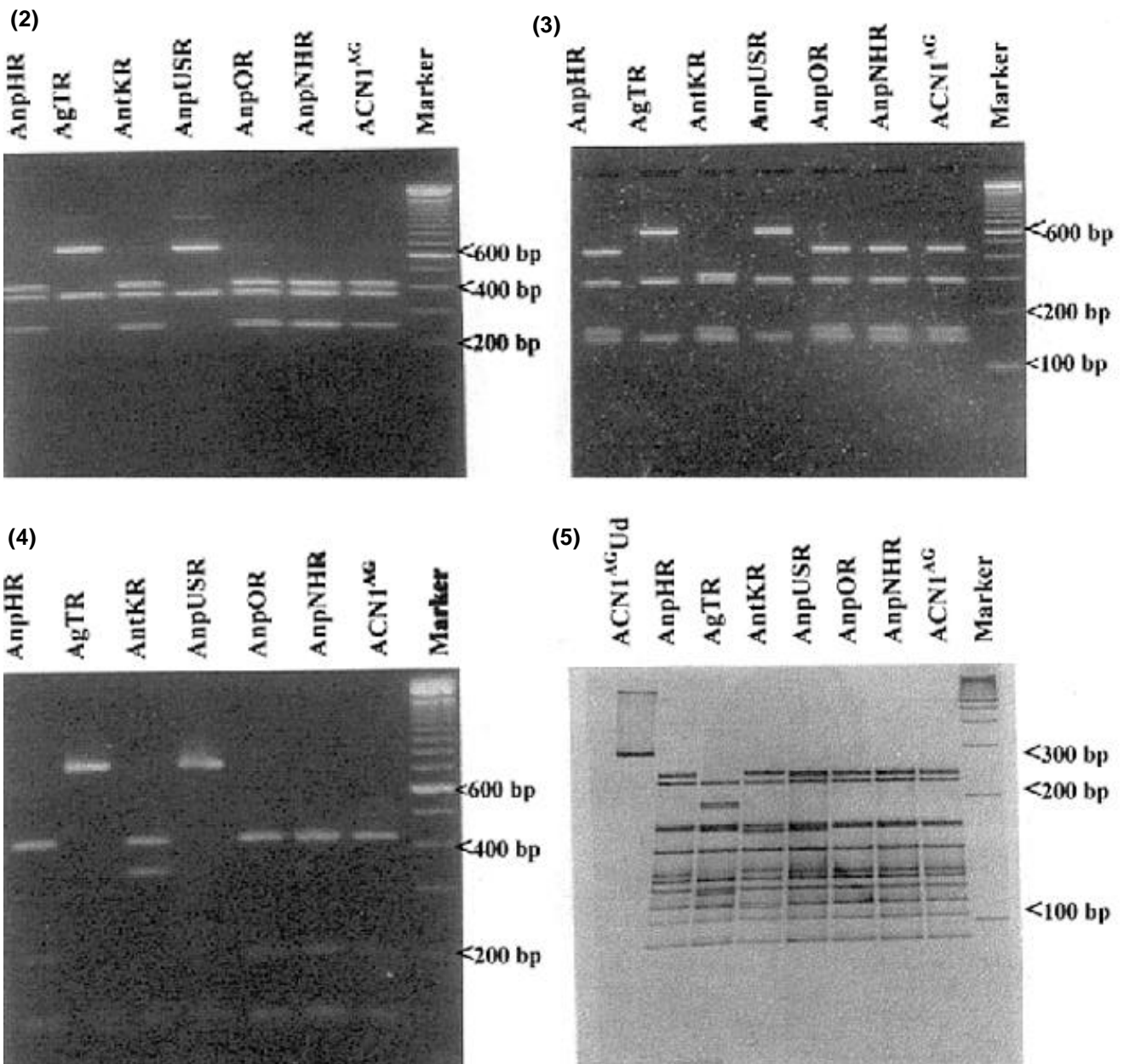
3. Results and discussion

3.1 Restriction fragment length profiles of *Frankia* ITS

The four restriction enzymes used (*TaqI*, *AvaI*, *HinfI* and *RsaI*) generated five different patterns for the 16S-23S rRNA ITS region. The restriction patterns obtained on digestion of 16S-23S rRNA ITS with *AvaI* (figure 2), *TaqI* (figure 3) and *HinfI* (figure 4) were similar for AnpHR, AnpOR, AnpNHR and ACN1^{AG}. While the first three were samples obtained from nodules of *A. nepalensis*, the last one was an isolate from the nodules of *A. crispa*. The pattern for AntKR from *A. nitida* nodules, was unique for digestion with *TaqI* and *HinfI*, but similar to the other four for digestion with *AvaI*. However, in all the cases the patterns for AnpUSR, the sample from nodule of *A. nepalensis* collected from upper Shillong, were similar to the patterns obtained for AgTR from nodule of *A. glutinosa* collected from Tuebingen, Ger-

many (figure 2–4). However, silver stained gels of 16S-23S rRNA ITS regions digested with *RsaI* could discriminate between AgTR and AnpUSR (figure 5). It may be noted that the samples AnpNHR and AnpUSR, that showed very different restriction patterns, were collected from two locations around Shillong that were just a few kilometers apart. On the contrary, the two samples, AnpUSR and AgTR, collected from two different *Alnus* species growing in two different continents, had similar patterns, except for the digestion with *RsaI*.

A similar experiment done on the *Casuarina equisetifolia* nodules collected from various parts of the world, including India, was able to demonstrate the presence of group I genotypes of *Frankia* outside Australia (Simonet *et al* 1999), with one exception. The senior author (unpublished) found this exception of Kenyan nodule samples referred to by Simonet *et al* (1999). Chauhan and Misra (2002) have used this technique for designing molecular markers for rejecting genotypes of alder with low nitrogenase activity of their actinorhizal root nodules.



Figures 2–5. Restriction patterns of 16S-23S rDNA ITS of *Frankia* amplicons from different hosts using *AvaI* (2), *TaqI* (3), *HinfI* (4) and *RsaI* (5).

3.2 Designing alder specific PCR primer

Alignment of the *A. nepalensis* 18S-28S ITS sequences with sequences of other species of alder, *Corylus*, *Carpinus* and *Betula* (retrieved from the GenBank) showed regions where all the samples analysed were similar, as well as regions where the alder sequences were similar, while the non-alder genera showed differences. One such site was found at 94–110 bp downstream of the *A. nepalensis* sequence in the distal region of the 18S rRNA gene and initial part of the ITS1. The primer designed in the present study was located here (code named primer AGPS24VC, 5'CCGCGAACCTGTCACAACAA3'). The 3' end of this primer, being located in the ITS1, had differences with the sequences of *Corylus*, *Carpinus* and *Betula*. Therefore, the chances of the primer annealing and amplifying these templates were small, especially if the stringency conditions were high. This primer in conjunction with primer ITSC26A was utilized for amplifying the two internally transcribed spacers and the 5-8S rRNA gene lying between the 18S and 28S rRNA genes of the nuclear DNA (figure 1).

Amplification of the 18S-28S rRNA region, using primer pair AGPS24VC/ITSC26A yielded the expected band of approximately seven hundred base pairs at an annealing temperature of 45°C. In addition to this band, other smaller bands were also present. One of the probable reasons for the occurrence of the additional bands was the presence of alternate annealing sites for the primer. The other reason could have been the presence of exogenous DNA of fungal origin. This was quite likely since several fungal hyphae and spores are normally present on leaf surfaces. It was for this reason, very young leaves were taken for isolation of DNA. Moreover, care was taken to thoroughly surface sterilize the leaves

with 30% H₂O₂ prior to isolation of DNA. However, to confirm that the contaminating fungal DNA, if any, did not get amplified, following experiment was conducted.

Fungal DNA and alder DNA were amplified using the primers AGPS24VC/ITSC26A under identical conditions at 45°C annealing temperature. Electrophoresis of the amplicons revealed several bands for alder DNA. The fungal DNA also showed multiple bands but these were fainter than those of alder. Therefore, various annealing temperatures from 50°C to 60°C were tried. At 52°C, a single strong band of approximately 700 bp was produced for alder and the fungal DNA did not amplify. Subsequent experiments with this primer pair were, therefore, carried out at an annealing temperature of 52°C.

Total genomic DNA was isolated from the leaves of all the genera tested. All the DNAs were amplified under identical conditions along with alder DNA. To be sure of the results a negative control was always kept. It was observed that alder samples got amplified but *Elaeagnus*, *Myrica*, *Betula* and *Carpinus* did not show any amplification (figure 6). Thus, on the basis of this experiment we can say that primer AGPS24VC is *A. nepalensis*-specific at the defined annealing temperature of 52°C. Perusal of aligned sequences of *A. nepalensis* with other species of the genus show that the primer should amplify the DNA of other members of this genus as well. However, other species of *Alnus* remain to be tested before declaring this primer as truly genus-specific.

4. Conclusion

In conclusion, we demonstrate the importance of the *rrn* ITS for designing specific probes and marker profiles for actinorhizal symbiosis. We were able to develop a PCR-

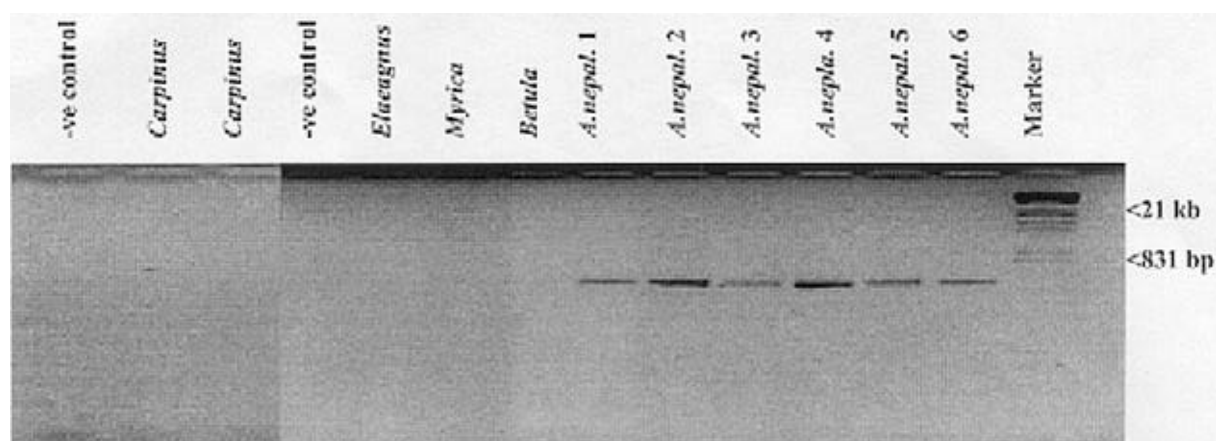


Figure 6. Amplification of DNAs of different trees using primers AGPS24VC and ITSC26A at 52°C annealing temperature. Gel documented by GelDoc1000 (BioRad, USA).

RFLP-based approach for *rrn* operon ITS region distinguishing between different strains of *Frankia* harboured by alders. The multisite restriction profile approach employed by us was able to differentiate between very similar endosymbionts of alder. We have also used the sequence variability of the nuclear *rrn* operon ITS regions of alder for designing alder-specific PCR primer. Taking advantage of the conserved genes, we were able to amplify the *A. nepalensis* DNA, about which no sequence information was available. This amplicon was then sequenced and the specific primer was designed for amplification of *A. nepalensis*, partly utilizing the hypervariable ITS region. Therefore, a similar approach for designing specific molecular markers for other symbiotic systems is recommended.

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