ISSN. 0972 - 8406

The NEHU Journal, Vol XV, No. 1, January-June 2017, pp.17-24

Study of Molecular Diversity of Un-isolated *Frankia alni* Present in Nitrogen-fixing Actinorhizal Nodules from India and Germany

RAJANI VARGHESE[1] & ARVIND K. MISRA[2]

Abstract

Actinorhizal plants contribute substantially to the nitrogen status of forest soils by way of harbouring nitrogen-fixing actinomycete Frankia *in their root nodules. It is expected that the nitrogen status of forest soils can be improved by selecting ideal host-microsymbiont combination with high nitrogen-fixing efficiency. Assessment of genetic diversity prior to selection of superior microsymbiont is a prerequisite. Therefore, to assess the quantum of natural variability, we report here screening of several* Frankia alni *samples present in actinorhizal nodules of genus* Alnus *for diversity at molecular level by targeting the ribosomal RNA operon.*

Keywords:*Frankia*, actinorhizal, *Alnus*, molecular diversity

Introduction

The ability to fix atmospheric dinitrogen to a usable form is a charac teristic property of some prokaryotes, in general called as nitrogen-fixers. There are two categories of nitrogen-fixing microorganisms. One group enters into a symbiotic association with some plants, while the other does not. *Rhizobium* sp. forms symbiotic nitrogen-fixing nodules with leguminous plants, while *Frankia* forms similar nodules with about 25 species of dicotyledonous forest trees belonging to eight families of plants. These trees are collectively called as actinorhizal trees.

[a1]Dr. Rajani Varghese is at the Ohio State University Medical Centre, Martha Morehouse Medical Plaza, Columbus, Ohio 43221, USA

[2]Prof. Arvind K. Misra teaches at the Department of Botany, North-Eastern Hill University, Shillong 793 022, India

[2]Corresponding author: Prof. Arvind K. Misra (arvindkmisra@gmail.com)

Frankia is an actinomycete distinguishable on the basis of three characteristic morphological features. These features are the septate hyphae, non-motile spores contained in sporangia (Lechevalier and Lechevalier, 1979) and pedunculate thick walled specialized structures called as vesicles (Lalonde and Calvert, 1979). The vesicles are sites of nitrogen fixation and contain the nitrogenase enzyme (Meesters *et al.*, 1987). Since this enzyme is oxygen sensitive, it requires extensive system to protect it. Vesicles are designed for this purpose. However, *Casuarina* compatible *Frankia* do not form vesicles inside the host. In this case the host cells form very thick cell walls.

Among the actinorhizal genera, *Alnus* and *Casuarina* exhibit highest nitrogen-fixing potential (Dommergues, 1996). Two species of *Alnus*, *A. nepalensis* and *A. nitida*, are found in India. While *A. nepalensis* is extensively found in eastern Himalayas extending into hills of northeastern India, *A. nitida* is found in western Himalayas. *A. nepalensis* trees are also found in Nilgiri Hills of peninsular India. *Alnus* plays an important role in forest ecology by way of harbouring nitrogen-fixing *Frankia* in its nodules, thereby enriching forest soils. Not only this, it also releases phenols, fatty acids and amino acids into forest soils that stimulate the growth of other soil bacteria.

The nitrogen-fixing potential of *Alnus* trees can be further enhanced by selecting superior strains of both host and its micro-symbiont (Verghese and Misra, 2000, 2002; Chauhan and Misra, 2002). Study of natural diversity of both the host and its micro-symbiont is the first step towards this aim. We report here the study of molecular diversity of *Frankia* found in *Alnus* nodules collected from six different locations.

Materials and methods

Nodule samples were collected from five locations spreading across India and one location in Germany (Table 1). Strain ACN1^{AG} was included as reference. DNA was isolated from nodules using the method of Rouvier *et al.* (1996), while the method described by Simonet *et al.* (1985) was used for isolation of DNA from reference strain. The purity of isolated DNA was checked using spectrophotometric optical density ratio of A_{260}/A_{280} and was visualised on 0.8% agarose gels. Purified DNA was subjected to Polymerase Chain Reaction (PCR) for amplification of 16S-23S rDNA ITS region as described earlier (Varghese *et al*. 2003a). The amplicon thus obtained was used as a probe for Southern hybridization of total genomic DNA digested with *Eco*RI/ *Hind* III and transferred to nylon membrane by capillary transfer method at neutral pH using 20 X saline sodium citrate (Southern, 1975). In order to carry out sequencing, the PCR amplified rDNA fragments were ligated into vector pUC18 at *Sma* I site and were cloned in *E. coli*. Positive clones were isolated from overnight cultures. The Expand Polymerase$^{(R)}$ was used for amplification of DNA to be used for sequencing reaction. The amplicon was purified using QIA quick purification

kit and sequenced using T7 sequencing kit (Pharmacia) and denaturing polyacrylamide gel. The sequences thus obtained were submitted to GenBank database (Table 1) and were aligned using CLUSTAL W (Thompson *et al*., 1994) with related sequence ORS020606 retrieved from GenBank. Phylogenetic tree was constructed from aligned sequences using PHYLIP for Windows (version 3.5c, Felsenstein, 1993).

Results and discussion

The isolated DNA produced a clean ~21Kb band in the agarose gel (Fig. 1) for all samples. The DNA amplification product for the 16S-23S rRNA ITS was consistently obtained in all cases as a single band of ~500 bp (Fig. 2). When amplified DNA was used as a probe for Southern hybridization, instead of one hybridization signal, two signals were obtained (Fig. 3). This could be because of either or both of the following reasons:

i) Partial digestion of the genomic DNA may have resulted in two products of varying sizes,

ii) Since *Frankia* genome contains two *rrn* operons (Normand *et al*., 1992), variation in these operons could yield two signals.

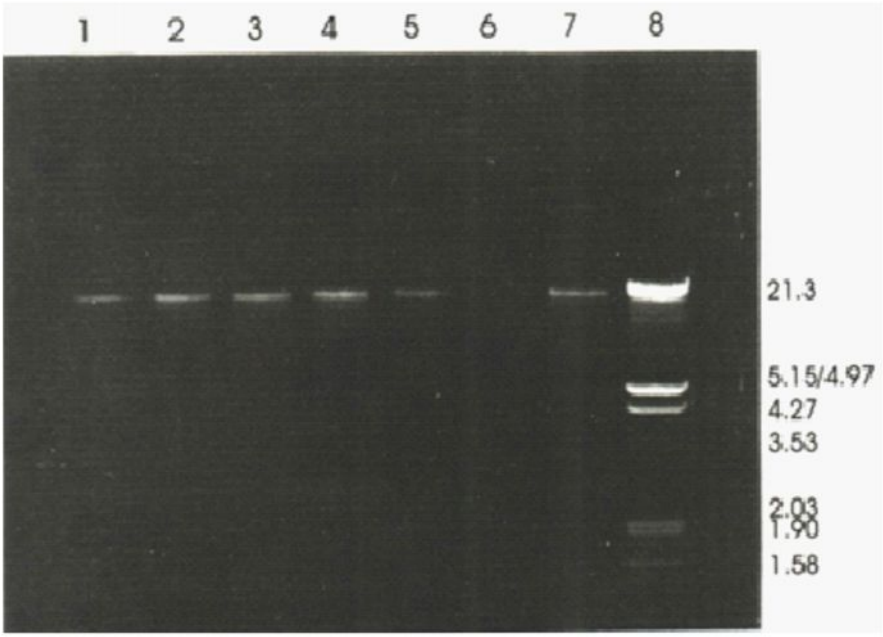

Fig. 1. Ethidium Bromide stained agarose gel showing clean bands of about 21 kb genomic DNA. Lane 1- Anarh, lane 2- Aggtun, lane 3- Anhpk, lane 4- Anmus, lane 5- Anob, lane 6- Anmnh, lane 7- ACN1^{AG} and lane 8- ? DNA Eco RI/ Hind III double digest as molecular marker.

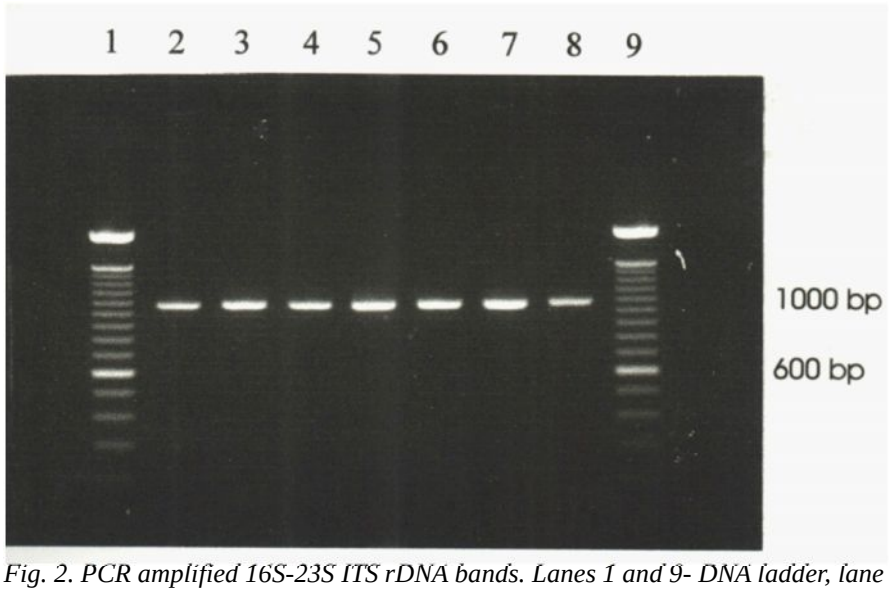

Fig. 2. PCR amplified 16S-23S ITS rDNA bands. Lanes 1 and 9- DNA ladder, lane 2- Anarh, lane 3- Aggtun, lane 4- Anhpk, lane 5- Anmus, lane 6- Anob, lane 7- Anmnh, and lane 8- ACN1^{AG}.

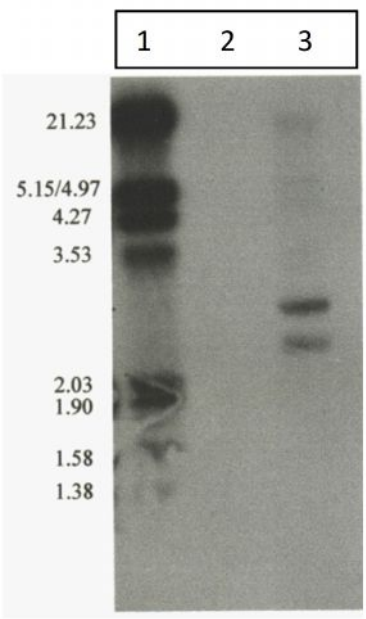

Fig. 3. Dig-11 UTP labelled probe signal for 16S-23S rDNA. Lane 1- ? DNA Eco RI/ Hind III double digest as molecular marker, lane 2- Aggtun, lane 3- Anmus

The amplified DNA for 16S-23S ITS region yielded clean sequence autoradiogram (Fig. 4). Regions of variability have been highlighted by putting * marks in the Fig. 4. The sequences obtained were deposited in GenBank data base (Table 1) and subjected to phylogenetic analysis by using neighbour joining method (Kimura 2 parameter) tree (Fig. 5). Most noticeable observation was that the *Frankia alni* present in the nodule sample from *A. nepalensis* tree in Upper Shillong was closely related to the *Frankia alni* present in nodule sample collected from *A. glutinosa* tree from Tuebingen, Germany. The implications of this have been explained by Varghese *et al.* (2003b). Another striking feature was that *Frankia* present in these two samples were closer to reference strains ACN1^{AG}, which was originally isolated in Canada from *Alnus viridis* subsp *crispa*, and ORS020606, an isolate from *Casuarina sp.* This cluster, supported by bootstrap value of 100, included *Frankia* from very diverse geographic regions as well as hosts.

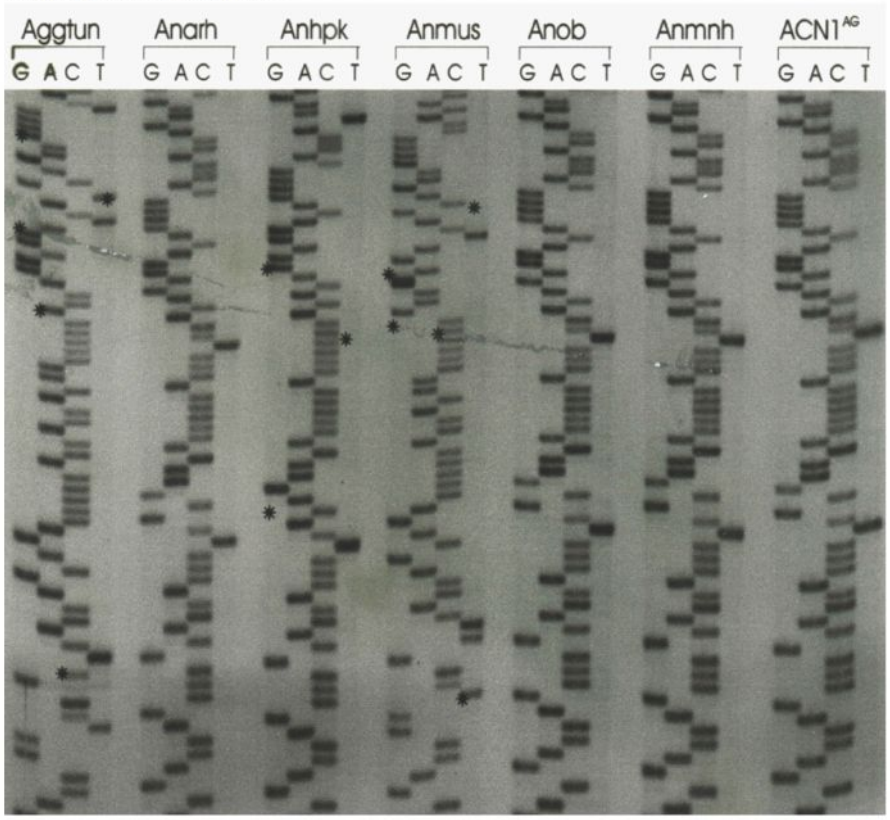

*Fig. 4. Autoradiogram of a sequencing gel showing sequence variations in the 16S-23S ITS region. Some of the differences have been highlighted by putting * marks.*

Table 1. List of nodule samples collected from different locations and GenBank accession numbers assigned to the corresponding rDNA ITS nucleotide sequences.

Location			Host species	Code assigned	GenBank Acc. No.
Country	State	Site			
India	Arunachal Pradesh	Hapoli	*Alnus nepalensis*	Anarh	AJ404866
	Meghalaya	Upper Shillong	*Alnus nepalensis*	Anmus	AJ404869
		Nonkrem Hills	*Alnus nepalensis*	Anmnh	AJ404867
	Tamil Nadu	Ooty	*Alnus nepalensis*	Anob	AJ404868
	Himachal Pradesh	Kulu	*Alnus nitida*	Anhpk	AJ404870
Germany		Tuebingen	*Alnus glutinosa*	Aggtun	AJ404871

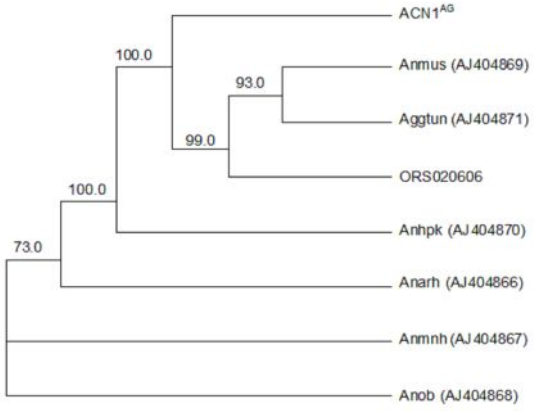

Figure 5. Neighbour-joining tree derived from aligned sequences of the 16S-23S ITS region. Figures at the forks indicate bootstrap values. Figures in parenthesis represent GenBank accession number.

This indicates that *Frankia* present in these nodules moved to different geographic zone and started nodulating different hosts, but did not diversify much from the original progenitor. On the contrary, the *Frankia* present in nodule sample collected from *A. nepalensis* from Nonkrem Hills at close proximity to Upper Shillong site was very different. Possibly this variant of *Frankia* diversified during the intervening period. Overall the tree showed presence of considerable genetic diversity among *Frankia* studied. It seems that *Frankia* has been evolving in India for a considerable period of time since it entered into symbiotic association with *Alnus* sp. The quantum of diversity could provide a good starting base for future selection of superior *Frankia alni* strains.

Acknowledgement

We are thankful to Prof. D. Ammerman and Dr. G. Steinbruck, University of Tuebingen, Germany for providing laboratory facilities to carry out part of this work.

References

Chauhan, V.S. and Misra, A.K. 2002. Development of molecular markers for screening *Alnus nepalensis* (D. Don) genotypes for the nitrogenase activity of the actinorhizal root nodules. *Molecular Genetics and Genomics* 267(3), 303-312.

Dommergues, Y.R. 1996. Nitrogen fixation in actinorhizal plants: Application. *Acta Botanica Gallica* 143, 663-679.

Felsenstein, J. 1993. PHYLIP (phylogeny inference package), Version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seatle, USA.

Lalonde, M. and Calvert, H.E. 1979. Production of *Frankia* hyphae and spores as infective inoculants for *Alnus* species. In: Gordon, J.C., Wheeler, C.T. and Perry, D.A. (Eds.), *Symbiotic Nitrogen Fixation in Management of Temperate Forests*, Oregon State University Press, Corvallis, USA, pp. 95-110.

Lechevalier, M.P. and Lechevalier, H.A. 1979. The taxonomic position of Actinomycete endophytes. In: Gordon J.C., Wheeler, C.T. and Perry, D.A. (Eds.), *Symbiotic Nitrogen Fixation in Management of Temperate Forests*, Oregon State University Press, Corvallis, USA, pp. 111-122.

Meesters T.M., Van Vliet W.M. and Akkerman A.D.L. 1987. Nitrogenase is restricted to the vesicles of *Frankia* strain EAN1pec. *Physiologia Plantarum* 70, 267-271.

Normand, P., Cournoyer B., Simonet P. and Nazaret S. 1992. Analysis of ribosomal operon in the actinomycete *Frankia*. *Gene* 111, 119-124.

Rouvier, C., Prin Y., Reddel P., Normand P. and Simonet P. 1996. Genetic diversity among *Frankia* strains nodulating members of the family Casuarinaceae in Australia revealed by PCR and restriction fragment length polymorphism analysis with crushed nodules. *Applied and Environmental Microbiology* 62, 979-985.

Simonet, P. Normand, P., Moiroud, A. and Lalonde, M. 1985. Restriction enzyme digestion patterns of *Frankia* plasmids. *Plant and Soil*87, 49-60.

Southern, E.M. 1975. Detection of specific sequences among DNA fragment separated by gel electrophoresis. *Journal of Molecular Biology* 98(3), 503-517.

Thompson, J.D., Higgins D.G. and Gibson T.J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting position specific gap penalties and weight matrix choice. *Nucleic Acid Research* 22, 4673-4680.

Varghese R., Chauhan, V.S. and Misra A.K. 2003a. Hypervariable spacer regions are good sites for developing specific PCR-RFLP markers and PCR primers for screening actinorhizal symbionts. *Journal of Biosciences,* 48(4), 437-442.

Varghese, R., Chauhan, V.S. and Misra, A.K. 2003b. Evolutionary implications of nucleotide sequence relatedness between *Alnus nepalensis* and *Alnus glutinosa* and between corresponding *Frankia* micro-symbionts. *Plant and Soil* 254, 219-227.

Verghese, S.K. and Misra, A.K. 2000. PCR-RFLP based screening of *Frankia* genotypes in alder nodules having different levels of nitrogenase activity. *Symbiosis* 28, 337-350.

Verghese, S.K. and Misra, A.K. 2002. *Frankia*-Actinorhizal symbiosis with special reference to host-microsymbiont relationship. *Current Science* 83(4), 404-408.