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Morphological and molecular characterization of *Frankia* sp. isolates from nodules of *Alnus nepalensis* Don.

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Abstract. Nodules collected from *Alnus nepalensis* growing in mixed forest stands at three different sites around Shillong, were crushed in various culture media to obtain isolates of *Frankia*. The isolates were found to have typical *Frankia* morphology as revealed by the scanning electron microscope. Seedlings inoculated with isolates or crushed nodules formed nitrogen fixing nodules. *Frankia* specific DNA probes amplified the DNA of the tested isolate AnpUS4. Partial nucleotide sequence of the 16S rRNA gene indicated that AnpUS4 was phylogenetically distinct from all other *Frankia* strains characterized so far.

Key words: *Frankia* sp. – *Alnus nepalensis* – SEM – PCR – Nucleotide sequence

Frankia isolates have been obtained from several actinorhizal plants using various methods and culture media ever since Callaham et al. (1978) first reported successful isolation of *Frankia* sp. from nodules of *Comptonia peregrina* (for references see Ganesh 1993). Morphology, chemistry, serology, DNA homology, determination of 16S rDNA sequence and demonstration of infectivity for host plant etc. have all been used as criteria for classifying a given actinomycete as *Frankia* (Simonet et al. 1991). Recently Frankiaceae, *Frankia* genus (Simonet et al. 1991), *Alnus* compatible frankiae and *Elaeagnus* frankiae specific (Bosco et al. 1992) DNA probes have been developed for specific amplification of DNA. Determination of the nucleotide sequence of part of the 16S rRNA gene has become a customary way to ascribe a new isolate to a given taxon, following the pioneering work of Woese (1987). Since 16S rDNA sequences of *Frankia* strains are now available (Nazaret et al. 1991) it has become a standard technique for relating *Frankia* isolates and even unisolated endophytes (Nick et al. 1992).

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We report here first isolates of *Frankia* sp. from nodules of *Alnus nepalensis*. We have used morphology, as revealed by scanning electron microscopy (SEM), host infectivity and DNA amplification using specific probes in the 16S rRNA region as tests for confirming the identity of the local isolates. Partial nucleotide sequence analysis has been employed to place the isolate tested in a phylogenetic tree.

Materials and methods

Isolation of Frankia from nodules

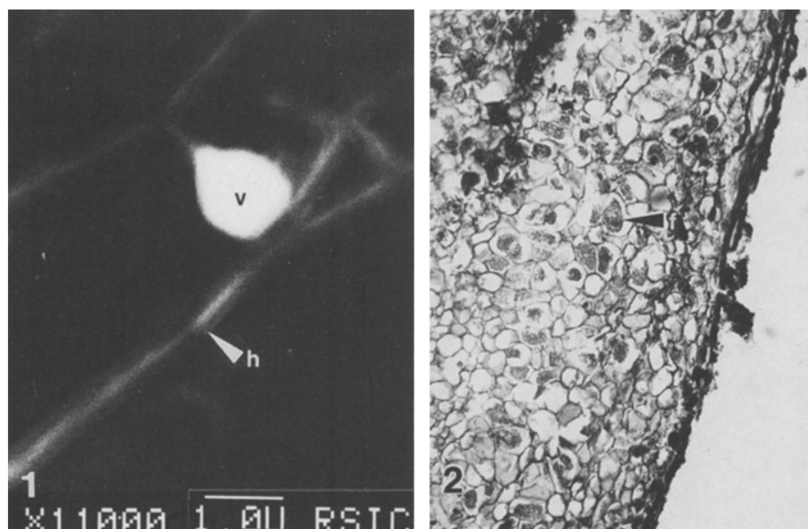
Nodules were collected from roots of *Alnus nepalensis* trees naturally growing at three different sites (Upper Shillong, Smit and Alugodam) around Shillong, India. Surface sterilized nodules were crushed on the walls of culture tubes containing various liquid media [defined propionate medium (DPM) (Baker and O'Keefe 1984), Qmod (Lalonde and Calvert 1979), OS-1 (Dobritsa and Stupar 1989) and F (Simonet et al. 1985)]. These tubes were incubated in dark at 28 ± 3 °C without agitation. Visible biomass appeared in about 3–4 weeks in some tubes. For uniformity, all isolates/strains were subsequently maintained in DPM throughout this study.

Scanning electron microscopy

Four isolates, representing the three sites, were studied under SEM Isolates/strains were fixed in 3% glutaraldehyde, washed in PS buffer (0.15 M sodium chloride, 0.01 M sodium phosphate, pH 7.4), stained with osmium tetraoxide, dehydrated in acetone series, dried to a critical point and gold sputtered before observing under SEM. The four isolates studied were named AnpUS4, AnpUS8, AnpST11 and AnpAG14. ARgP5, ACN1^{AG} and ArI3 were used as reference strains.

Host plant root infectivity

Fifteen days old seedlings of *A. nepalensis*, grown at room temperature under sterile conditions were inoculated for root infectivity studies. Ten to fifteen seedlings per inoculum were tested in two replicates. They were fed nitrogen free Hoagland solution (Hoagland and Arnon 1950) and monitored for nodulations. Appropriate positive and negative controls were maintained.



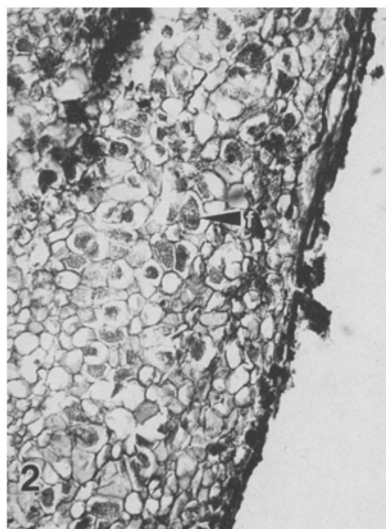


Fig. 1. Scanning electron micrograph of AnpAG14, showing vesicle (v) and hyphae (h)

Fig. 2. TS of root nodule of *Alnus nepalensis* seedling inoculated with strain AnpUS8 showing *Frankia* (f) in the cortical region

DNA amplification

One of the isolates was subjected to DNA amplification using Frankiaceae (FGPS958, Simonet et al. 1991) and *Alnus-Casuarina* compatibility groups specific (FGPS989ac, Bosco et al. 1992) probes for the 16S rRNA gene. Sonicated culture or purified DNA were directly used for amplification as described earlier (Simonet et al. 1991). DNA amplification for a larger segment was achieved using universal prokaryote specific primers FGPS849 and FGPS1176' (Simonet et al. 1991).

Determination and analysis of nucleotide sequence

The DNA amplified using probes FGPS849 and FGPS1176' was purified using GeneClean II kit (BIO 101, USA) and sequenced as described earlier (Nazaret et al. 1991). Sequences were aligned and compared with Nazaret et al. (1991) alignment of several *Frankia* strains using the Clustal programme of Higgins and Sharp (1988). For each pair of sequences, the number of substitutions was calculated. A phylogenetic tree was obtained using the Neighbour-Joining distance method of Saitou and Nei (1987).

Results and discussion

Almost 50% of the tubes not contaminated (i.e. those that did not develop turbidity) showed visible biomass. The colonies were whitish to yellowish, compact and settled at the bottom. Microscopic examination revealed *Frankia* like colonies with characteristic features such as vesicles.

All isolates showed thin, highly branched hyphae and vesicles similar to the reference strains (Fig. 1). Hyphal

	1	11	21	31	41	51
1 AnpUS4	TTGACGGGGG	CCCGCACAAG	CGGCGGAGCA	TGTGGCTTAA	TTCGATGCAA	CGCGAAGAAC
2 Ari3	TTGACGGGGG	CCCGCACAAG	CGGCGGAGCA	TGTGGCTTAA	TTCGATGCAA	CGCGAAGAAC
3 Avn17o	TTGACGGGGG	CCCGCACAAG	CGGCGGAGCA	TGTGGCTTAA	TTCGATGCAA	CGCGAAGAAC
	61	71	81	91	101	111
1 AnpUS4	CTTACCTAGG	CTTGACATGC	ACGGAAATCC	TCCAGAGATG	GTGGGT-CCG	CAAGGGTCCT
2 Ari3	CTTACCAGGG	CTTGACATGC	AGGGAAATCT	CGTAGAGATA	CGGGGT-CCG	TAAGGGTCCT
3 Avn17o	CTTACCAGGG	TTTGACATGC	AGGGAAATCC	TCCAGAGATG	GGGGGT-CCG	TAAGGGTCCT
	121	131	141	151	161	171
1 AnpUS4	GCACAGGTGG	TGCATGGTTG	TCGTCAGCTC	GTGTCGTGAG	ATGTTGGGTT	AAGTCCCGCA
2 Ari3	GCACAGGTGG	TGCATGGCTG	TCGTCAGCTC	GTGTCGTGAG	ATGTTGGGTT	AAGTCCCGCA
3 Avn17o	GCACAGGTGG	TGCATGGCTG	TCGTCAGCTC	GTGTCGTGAG	ATGTTGGGTT	AAGTCCCGCA
	181	191	201	211	221	231
1 AnpUS4	ACGAGCGCAA	CCCTCGTTCT	ATGTTGCCAG	CAC--GTTAT	G----GTGGG	GACTCATAGG
2 Ari3	ACGAGCGCAA	CCCTCGTCCT	ATGTTGCCAG	CGA--GTTAT	G----TCGGG	GACTCATAGG
3 Avn17o	ACGAGCGCAA	CCCTCGTCCT	ATGTTGCCAG	CGA--GTAAT	G----TCGGG	GACTCATAGG
	241	251	261	271		
1 AnpUS4	AGACTGCCGG	GGTCAACTCG	GAGGAAGGTG	GGGAT		
2 Ari3	AGACTGCCGG	GGTCAACTCG	GAGGAAGGTG	GGGAT		
3 Avn17o	AGACTGCCGG	GGTCAACTCG	GAGGAAGGTG	GGGAT		

Fig. 3. Partial sequence alignment of 16S rRNA gene of strain AnpUS4 with one closely and one distantly related strains

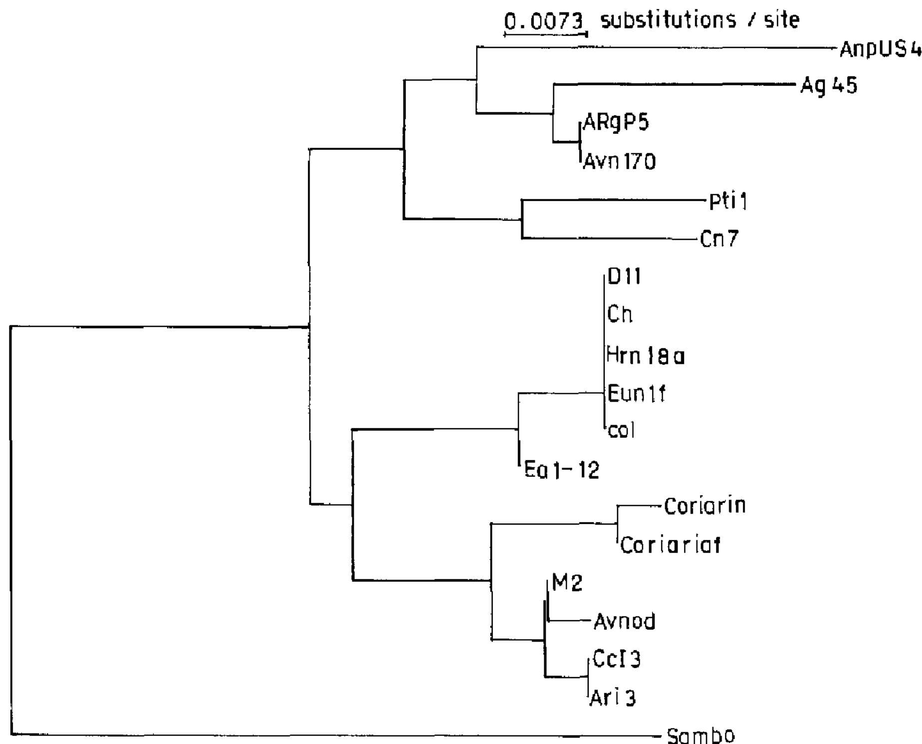


Fig. 4. Neighbour-joining tree aligned 16S sequences given in Fig. 3. *S. ambofaciens* sequence was found to have 0.111 substitutions per site with respect to the *Frankia* strains

diameter ranged between 0.3–1.0 μm and the vesicle diameter ranged between 0.6–4.0 μm.

Observations on seedlings inoculated with the isolates/strains under study showed root hair deformations within ten days, which were similar to the ones reported by Margheri et al. (1989). Pre-nodules (Torrey and Callaham 1979) were seen in about four weeks and true nodules were formed in about six weeks after inoculation. Nodulated seedlings as well as those with pre-nodules showed nitrogenase activity as revealed by acetylene reduction assay. Nodulated seedlings were sectioned using standard microtomic techniques and the endophyte was seen in the cortical region of the sectioned nodules (Fig. 2).

The isolate subjected to DNA amplification using Frankiaceae and *Alnus-Casuarina* compatibility frankiae groups specific probes responded positively to both, producing amplified DNA of expected sizes. This isolate, thus, can safely be placed in the family Frankiaceae (Simonet et al. 1991) as also in the genus *Frankia*, belonging to *Alnus-Casuarina* specificity group of Bosco et al. (1992).

The partial sequence of the 16S rRNA gene of isolate AnpUS4 was found to be unique, and relatively distanced from all known *Frankia* isolates (Fig. 3). The closest relatives appear to be ARgP5 and AVN17o (Fig. 4), which constitute two outlying genomic species infective on alder (Fernandez et al. 1989). Another neighbour would be Ag45, thus constituting a group of divergent *Alnus* infective *Frankia* strains. There are a number of sequence positions where AnpUS4 sequence is closer to *Elaeagnus* infective strains, others where it is closer to *Coriaria* infective strains and still others where it is unique, where strain specific primers could be targeted.

The differences in the sequence prompted us to confirm the presence of *nif* genes in the isolate. This was done by amplifying DNA using universal *nif*H, *nif*D and *nif*K probes yielding expected bands (results not shown).

Obviously, the isolates obtained from the nodules of *Alnus nepalensis* were strains of *Frankia*. The isolate tested was found to be genetically different from the reference strain, indicating the possibility of diversity amongst the frankiae of this region. This may not be surprising given the fact that few *Alnus* infective *Frankia* strains have been isolated and characterized from eastern Asia where genus *Alnus* is thought to have originated (Furlow 1979).

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