INVESTIGATIONS ON MOLECULAR DIVERSITY IN ALDER COMPATIBLE FRANKIAE

BY

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To my Father

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DECLARATION

I, Rajani Varghese, hereby declare that the subject matter of this thesis entitled "Investigations on molecular diversity in Alder compatible frankiae" is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to any body else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the North-Eastern Hill University for the award of the degree of Doctor of Philosophy in Botany.

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CHAPTER 1

INTRODUCTION

For several centuries it has been a common knowledge that the soils from which nitrogen has been removed by the cereal crops could be revitalized by growing leguminous plants. Yet it was during the beginning of the nineteenth century that adequate explanation started coming in about certain microorganisms that have the ability to fix atmospheric di-nitrogen to a more utilisable reduced form (NH_4^+). The ability to assimilate elementary nitrogen biologically by a comparatively few species of microorganisms is of great biological interest, specially since it is an important input to natural as well as agricultural eco-systems.

Biological nitrogen fixation in nature is brought about by two types of microorganisms - non-symbiotic or free living and symbiotic. Non-symbiotic microorganisms (e.g. certain cyanobacteria like *Rivularia*, *Scytonema* or free living bacteria like *Klebsiella*), fix nitrogen independent of a host, while, symbiotic microorganisms like *Rhizobium* and *Frankia* make use of a host plant for fixing atmospheric di-nitrogen (*Frankia* can fix nitrogen *ex planta* also). While *Rhizobium* symbiosis fixes 24-584 kg N $ha^{-1}y^{-1}$ of atmospheric nitrogen, the quantum of nitrogen fixation by actinorhizal plants is of the order of 2-362 kg N ha^{-} y^{-} (Shantharam and Mattoo, 1997).

The microsymbiont within the root nodules of actinorhizal plants was first described in the second half of the nineteenth century. Microorganisms that are able to induce nitrogen fixing root nodules on non-leguminous actinorhizal plants have been classified in the genus *Frankia* (Tjepkema and Torrey, 1979). The first successful isolation of *Frankia* CpI1 from *Comptonia peregrina* was done by

Callaham *et al.* (1978). Recently Normand *et al.* (1996) have classified *Frankia* as the only genus of the family *Frankiaceae*.

Members of the genus *Frankia* can be distinguished from other actinomycetes on the basis of their characteristic morphology, ability to nodulate plants and to fix nitrogen (although some strains of *Frankia* do not have the ability to nodulate or fix nitrogen). It is very typical in having high G+C content (about 68-72%; An *et al.*, 1983). Being a pleomorphic actinomycete, both *in vivo* and *in vitro* (Callaham *et al.*, 1978), *Frankia* exhibits three morphological structures. These are hyphae which are septate, sporangia containing non-motile spores (Lechevalier and Lechevalier, 1979) and pedunculate thick walled specialized structures called as 'vesicles' (Lalonde and Calvert, 1979). The enzyme nitrogenase is located in these symbiotic vesicles. Therefore, they are considered as the sites for nitrogenase activity *in vivo* and *in vitro* (Newcomb and Wood, 1987). However, *Casuarina* infective *Frankia* do not form vesicles inside the nodules. The nitrogenase is protected by the thickening of the host cell walls.

Frankia grows primarily by extension and branching of filamentous septate hyphae (~0.5-1.5 μm in diameter). Wider diameter hyphae differentiate into sporangia from which the spherical shaped asexual spores are released. Frankiae are able to use few carbon sources for energy and growth (Benson and Hanna, 1983; Hafeez *et al.*, 1984; Lechevalier *et al.*, 1983). Although, the exact nutritional requirement of *Frankia* is not fully understood, most isolates are able to use propionate, acetate, succinate, fumarate, malate and tween 80. They do not use hexoses, pentoses and disaccharide. Nitrogen utilization is restricted to ammonium, nitrite and aspartate (Shipton and Burggraf, 1982). The optimum temperature for growth is strain dependent ranging from 25°C-36°C (Tisa *et al.*, 1983).

Frankia has a varied host spectrum being in symbiosis with 25 genera of dicotyledonous plants belonging to 8 families (Baker and Schwintzer, 1990). Among actinorhizal plants, the two genera *Alnus* and *Casuarina* exhibit highest nitrogen fixing potential (Dommergues, 1996). The genus *Alnus* is one of the most extensively studied genus among actinorhizal plants. About 47 species of *Alnus* are known (Swensen and Mullin, 1997). In India, only two species of *Alnus* (*A. nepalensis* and *A. nitida*) are found distributed naturally throughout the temperate Himalaya. They are confined to the higher elevations of Meghalaya, Arunachal Pradesh and Himachal Pradesh. Although not naturally occurring, trees of *A. nepalensis* (fig 1.1) are also found in some locations of Nagaland and Tamilnadu. *A. nitida* trees (fig 1.2) are confined to higher altitudes of Himachal Pradesh (1200-1500 m above mean sea level), some time descending in to the plains along the river banks. They are morphologically quite distinct from *A. nepalensis* being larger in size, having lighter tree bark and bearing larger female cones. *Alnus glutinosa*, which was selected as an out species, in comparison is more or less like *A. nepalensis* or *A. nitida* in morphology except that its leaf blades are irregularly dentate (fig 1.3).

Alnus, together with its microsymbiont *Frankia*, is widely recognized as a good nitrogen fixer (Simonet *et al., 1991*; Guofan and Tingxiu, 1987; Domenach *et al.*, 1989). It is thought to be responsible for a high level of soil nitrogen accretion world wide (Tarrant and Trappe, 1971). In addition to this, *Alnus* plays an important role in forestry. It is cultivated in general for fuel woods or as timber or to increase soil nitrogen stability. Also, the release of compounds such as phenols, fatty acids, amino acids into soil indirectly affect the free living nitrogen fixing organisms stimulating their growth. In India, these trees are maintained in Himalayan uplands, used to colonize wasteland and to reclaim land to improve wild life habitat. Their economic value could be further explored and exploited by

Fig 1.1 : *Alnus nepalensis* trees as found growing in the Upper Shillong region of Meghalaya.

Fig 1.2 : *Alnus nitida* tree growing in Kulu, Himachal Pradesh

Fig 1.3 : *Alnus glutinosa* tree growing near the Neckar river bank of Tuebingen, Germany.

improving their nitrogen fixing efficiency. The nitrogen fixing potential can be significantly improved further genetically by selecting superior strains of *Frankia* and the corresponding host genotype for an enhanced symbiotic performance. In order to genetically improve any genus, initially its genetic diversity has to be screened. Following which selection of most competitive and infective strains could be brought about. This is necessary since, the ability to survive in soil even in absence of a host allows greater flexibility and diversity among frankiae strains (Swensen and Mullin., 1997).

Alnus is thought to have originated in Indo-China region (Furlow, 1979). Therefore, it is expected that its microsymbiont too would have evolved with it. If it is true, there should be higher diversity for *Frankia* in this region. This assumption is supported by the study conducted by Ganesh *et al.* (1994) who found that the isolate AnpUS4 generated from *Alnus nepalensis* was genetically different from the reference strains studied. But no detailed study is available yet on the diversity in alder compatible *Frankia* from different geographical locations in India. Therefore, in the present study conserved as well as variable regions of two very important structural and functional gene units (ribosomal and nitrogen fixing genes) from *Alnus* nodulating microsymbiont, collected from various geographical locations in India, were investigated.

The ribosomal DNA is well characterised ubiquitous molecular yardstick for estimating evolutionary relationship of organisms (Ochman and Wilson, 1987; Woese, 1987). In bacteria, the usual order of structure of *rrn* operon is 16S-spacer-23S-spacer-5S with few exceptions (fig 1.4). The ribosomal RNA genes are evolutionarily homologous and functionally equivalent in all organisms. Their sequence changes are slow allowing the estimation of evolutionary relationships between even distantly related organisms. The internal transcribed spacers (ITS) in prokaryotic rRNA genes are situated between 16S and 23S (fig 1.4) and between

23S and 5S genes. These are varied in length and are assumed to have been subjected to less selection pressure. Therefore, they should have accumulated more random mutations than the coding regions. Thus, it forms an ideal region for the discriminative studies and can be PCR amplified using specific primers corresponding to the flanking conserved sites.

One of the most important functions in *Frankia* is nitrogen fixation. The rate at which nitrogen is fixed by *Frankia* varies considerably depending upon plant species it is associated with (Torrey, 1978). The presence of nitrogen fixing genes in *Frankia* makes it economically important, thereby generating interest in the genetic study of the symbiosis. The nitrogenase enzyme involved in the fixation of the molecular nitrogen is a useful tool for characterising nitrogen fixing microorganisms. Nitrogenase is an enzyme complex consisting of two components - the molybdenum-iron protein called dinitrogenase, which is an $\alpha_2\beta_2$ tetramer encoded by *nif* D and *nif* K respectively and the iron containing protein called dinitrogenase reductase, which is a homodimer encoded by *nif* H (Hirsch, 1995). In addition to the structural genes (*nif* H, *nif* D and *nif* K; fig 1.5), biological nitrogen fixation requires a host of additional genes. In *Frankia*, a 4.5 kb fragment containing eight *nif* genes has been characterised (Harriott *et al* ., 1995) and till date more than twenty additional *nif* or *nif* associated genes have been identified in other diazotrophic bacteria (Oh *et al.*, 1997). In all cases studied it is found clustered in bacterial genome or plasmids (Merrick, 1993). Also, as in almost all nitrogen fixing organisms, *nif* H-D-K genes seem to be contiguous in *Frankia* (Haselkorn, 1986; Oh *et al.*, 1997). Large sequence homology has been established between *nif* H-D-K genes from *Klebsiella pneumoniae* (Cannon *et al.*, 1979) and those of other nitrogen fixing species (Ruvkun and Ausubel, 1980) including *Frankia.*

The nitrogenase structural genes (*nif* H, *nif* D and *nif* K) are known to carry both conserved (Ruvkun *et al.*, 1980) and variable regions present in low and

Fig 1.4 : Diagrammatic representation of organization of rrn operon in prokaryotes. The underlined segment marks the analysed region of the genes.

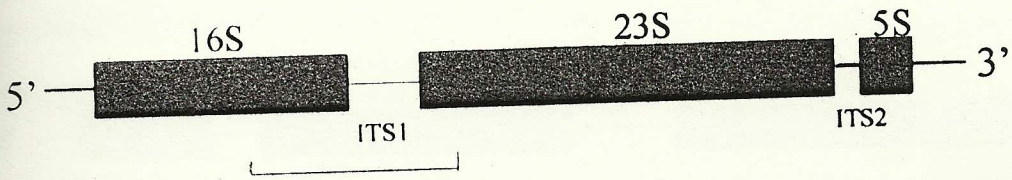
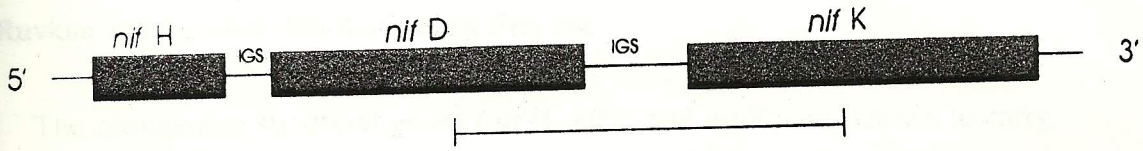

16S
23S
5S
5'
3'
ITS1
ITS2

Fig 1.5 : Schematic representation of the organization nitrogenase structural genes (*nif* H, *nif* D, *nif* K) genes. The line marked below represents the analysed region.


nif H
IGS
nif D
IGS
nif K
5'
3'

high sequence variability (Simonet *et al.*, 1990; Jamann *et al.*, 1993). Genes coding for nitrogenase and 16S rRNA are considered to have has been characterised (Harriott *et al* ., 1995) and till date more than twenty additional *nif* or *nif* associated genes have been identified in other diazotrophic bacteria (Oh *et al.*, 1997). In all cases studied it is found clustered in bacterial genome or plasmids (Merrick, 1993). Also, as in almost all nitrogen fixing organisms, *nif* H-D-K genes seem to be contiguous in *Frankia* (Haselkorn, 1986; Oh *et al.*, 1997). Large sequence homology has been established between *nif* H-D-K genes from *Klebsiella pneumoniae* (Cannon *et al.*, 1979) and those of other nitrogen fixing species (Ruvkun and Ausubel, 1980) including *Frankia.*

The nitrogenase structural genes (*nif* H, *nif* D and *nif* K) are known to carry both conserved (Ruvkun *et al.*, 1980) and variable regions present in low and high sequence variability (Simonet *et al.*, 1990; Jamann *et al.*, 1993). Genes coding for nitrogenase and 16S rRNA are considered to have evolved in parallel (Simonet *et al.*, 1991). Therefore, evolutionary studies on *Frankia* genome concentrate mostly on these two regions.

1.1 Objectives

Alnus is a largely studied genus from an ecological point of view. Very few investigations have been conducted at molecular level, especially in the Indian alder populations. Studies on *Frankia* in general, have shown existence of tremendous diversity (An *et al.*, 1985a; Mirza *et al.*, 1994; Navarro *et al.*, 1992; Rouvier *et al.*, 1996; Ritchie *et al.*, 1999). A superior and efficient host-microbe relationship could certainly affect positively the nitrogen fixing capacity of the *Frankia* strain. In order to accomplish this, selection of most infective, effective and competitive *Frankia* strain is necessary. For this, the diversity existing within the species has to be investigated which will indirectly enhance the prospect of genetically improving the

genus. Therefore, in this study, the existence of the diversity within *Frankia* nodulating *Alnus* species in India was investigated by molecular characterization of the microsymbiont directly inside the nodule. Since hidden diversity within the genus is important for the evolution and speciation of *Frankia* (Cournoyer *et al.*, 1993), the study conducted was also expected to put some light on the relationship existing among and between the species. The following approaches were tried for the purpose-

i). Alder compatible *Frankia* germplasm from different parts of India was collected. For comparative study, *Alnus glutinosa* nodules from Tuebingen, Germany, were also collected as an outgroup.

ii). DNA from field collected nodules and the reference *Frankia* strain $ACN1^{AG}$ were isolated.

iii). Polymerase Chain Reaction based amplifications of partial 16S rRNA gene, 16S-23S rDNA internal transcribed spacer (ITS) region, partial *nif* D and *nif* K genes and *nif* D-K intergenic spacer (IGS) regions, were done.

iv). Amplified Fragment Length Polymorphism was studied.

v). PCR/Restriction Fragment Length Polymorphism of 16S-23S rDNA ITS and *nif* D-K IGS regions were investigated.

vi). Partial 16S rRNA gene, 16S-23S rRNA ITS, partial *nif* D and *nif* K genes and *nif* D-K IGS regions were cloned and sequenced.

vii). Computer analyses of these sequences were done using the *Casuarina* compatible strain ORS020606 sequence retrieved from the GenBank as reference.

viii). Amino acid sequence of the partial *nif* D and *nif* K genes were developed and analysed using computers.

ix). Phylogenetic relationships among the sequences under study and those retrieved from the GenBank were worked out.